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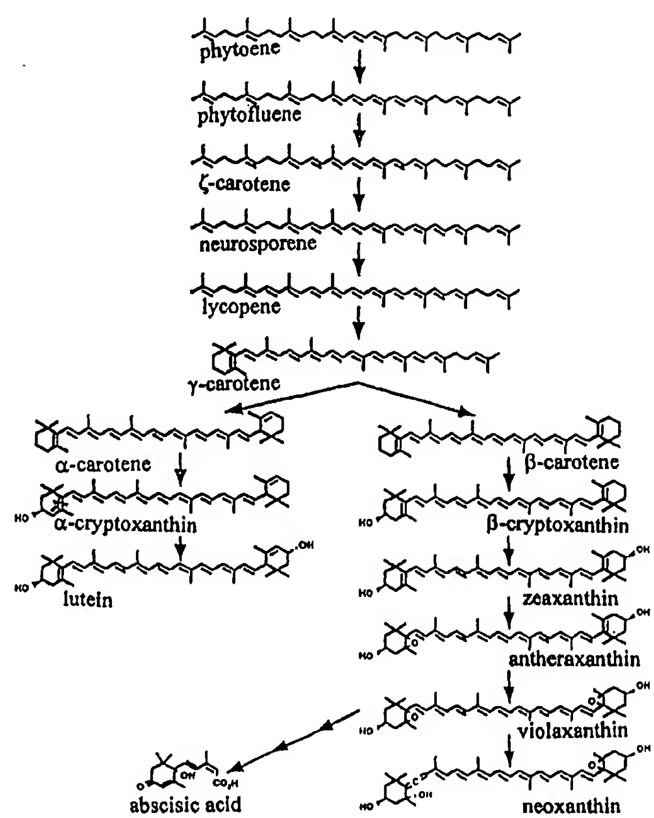
(54) Title: METHOD FOR REGULATING CAROTENOID BIOSYNTHESIS IN MARIGOLDS

(57) Abstract

A method for manipulating the ratio of various carotenoids in a plant as a means for augmenting the accumulation of selected carotenoids is described. Transgenic marigold plants which produce various ratios of carotenoids and methods for producing the same are described. Preferably, various carotenoids are accumulated in the petals of marigold by selecting a specific combination of isolated DNAs encoding various enzymes involved in the carotenoid biosynthesis pathway to produce antisense RNA, sense RNA or combinations thereof. Transgenic marigold which specifically accumulates carotenoids in the petals are described. Also described are isolated DNA sequences encoding the marigold genes beta-cyclase, epsilon-cyclase, beta-hydroxylase and isopentyl pyrophosphate isomerase.



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METHOD FOR REGULATING CAROTENOID BIOSYNTHESIS IN MARIGOLDS

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention provides a method for manipulating the ratio of various carotenoids in plants as a means for augmenting the accumulation of selected carotenoids. The present invention further relates to transgenic marigold plants which produce various ratios of carotenoids and methods for producing the same. Preferably, various carotenoids can be accumulated in the petals of marigold by selecting a specific combination of isolated DNAs encoding various enzymes involved in the carotenoid biosynthesis pathway to produce antisense RNA, sense RNA or combinations thereof. The present invention also describes isolated DNA sequences encoding the marigold genes beta-cyclase, epsilon-cyclase, beta-hydroxylase, isopentyl pyro-phosphate isomerase.

(2) Description of the Related Art

Carotenoids which comprise the most important group of 40-carbon terpenes and terpenoids are pigments that have a variety of commercial applications. Carotenoids are a class of hydrocarbons (carotenes) and their hydroxylated derivatives (xanthophylls) which comprise 40-carbon (C_{40}) terpenoids consisting of eight isoprenoid (C_5) units joined together. The terpenoids are joined in such a manner that the arrangement of the isoprenoid units is reversed at the center of the molecule placing the terminal methyl groups in a 1,6 relationship and the non-terminal methyl groups in a 1,5 relationship. Carotenoids can be monocyclic, bicyclic or acyclic. Carotenoids are produced by a wide variety of bacteria, fungi, and green plants. The carotenoids of the most value are intermediates in the carotenoid biosynthetic pathway and consist of lycopene (ψ,ψ-carotene), beta-carotene (β,β-carotene), zeaxanthin (β,β-carotene-3,3'-diol), astaxanthin (β,β-carotene-3,3'-diol-4,4'-diketo), lutein (β,ε-carotene-3,3'-diol) and alpha-carotene (β,ε-carotene).

Lycopene is a red carotenoid and has utility as a food colorant. Lycopene is naturally synthesised from the precursor phytoene through a series of four separate

dehydrogenation steps by the removal of eight atoms of hydrogen. Lycopene is an intermediate in the biosynthesis of other carotenoids in some bacteria, fungi, and all green plants.

Beta-carotene is an orange carotenoid that is naturally produced from lycopene through the intermediate gamma-carotene (β,ψ-carotene) by two sequential cyclization reactions that produce beta rings at the termini. Beta-carotene is useful as a colorant for margarine, butter and cheese, and as a provitamin which has been suggested to have a role in cancer prevention. Current commercial methods for producing beta-carotene include isolation from carrots, chemical synthesis and microbial production.

Zeaxanthin is a yellow carotenoid that is naturally produced from beta-carotene through the intermediate beta-cryptoxanthin by hydrogenation reactions which add hydroxyl groups to the beta rings at both termini. Zeaxanthin is used as a colorant in the poultry industry. Zeaxanthin can be synthesized chemically, however, current chemical synthesis reactions are inefficient and are not commercially competitive. Therefore, zeaxanthin is usually produced by extraction from corn grain, and corn gluten meal. However, all of these plant sources are characterized by low and inconsistent production levels.

Alpha-carotene is another yellow carotenoid that is naturally produced from lycopene through the intermediate δ-carotene (ε,ψ-carotene) by two sequential cyclization reactions at the termini that produces one terminus with an epsilon ring and the other terminus with a beta ring. Alpha-carotene is useful as a colorant and as a provitamin.

Carotenoids have a variety of commercial uses ranging from use as a pigment to color foods and cosmetics to uses by the pharmacological industry. Pharmacological uses include use as a control during manufacture to distinguish one drug product from another, as an active component of various medicinal compositions, and as a vitamin supplement for humans. Carotenoids are also used as a dietary supplement in animal and poultry feedstuffs. Carotenoids have even been used as a photoconductor in recording-media film.

In humans and animals carotenoids have diverse biological functions, and despite the similarity in structure, have different roles. Certain carotenoids are precursors to vitamin A

which can be converted to vitamin A by the body, examples are beta-carotene, alpha-carotene, and alpha-cryptoxanthin.

Aside from a role as a precursor to vitamin A, carotenoids are effective quenchers of oxygen free radicals, with lycopene exhibiting the highest quenching activity. Carotenoids function as chain-breaking antioxidants and therefore protect the body from damage by free radicals. Free radicals have been implemented in a wide range of human ailments such as onset of pre-mature aging, cancer, atherosclerosis, cataracts, and an array of degenerative diseases. Carotenoids have also been shown to enhance the immune system and to protect the skin from UV damage.

At present only a few plants are widely used to produce carotenoids. However, production of carotenoids from plants is expensive because of the low yields and variability of production. Recombinant DNA technology is a means for increasing the productive capacity of carotenoid biosynthesis in plants.

In U.S. Patent No. 5,429,939 to Misawa *et al* DNA segments from *Erwinia uredovora* encoding bacterial enzymes geranylgeryanyl pyrophosphate synthase, zeaxanthin glycosylase, lycopene cyclase, lycopene synthase, phytoene synthase, and beta-carotene hydroxylase are disclosed. The abovementioned U.S. Patent provides a process for producing a carotenoid or a precursor compound in a host but the invention does not provide a means for controlling the ratio of specific carotenoids in a plant.

In U.S. Patent No. 5,530,188 to Ausich *et al* DNA segments encoding *Erwinia herbicola* enzymes geranylgeryanyl pyrophosphate, phytoene synthase, phytoene dehydrogenase-4H, and lycopene cyclase are disclosed. The abovementioned patent provides a means for producing beta-carotene in a plant containing the DNA segment encoding lycopene cyclase. However, the U.S. Patent does not provide a means for controlling the ratio of specific carotenoids in a plant thereby producing plants that produce other valuable carotenoids.

In U.S. Patent No. 5,618,988 to Hauptmann *et al*, recombinant DNA technology was used to enhance carotenoid accumulation in the storage organs of genetically engineered plants by introducing into the plant a vector comprising a chimeric polypeptide consisting of the bacterial gene encoding phytoene synthase conjugated to a plastid transit peptide.

The phytoene synthase was derived from the bacterium *Erwinia herbicola*. While the abovementioned U.S. Patent provides a means for increasing production of phytoene which then serves as a precursor to pigmented carotenoids, the patent does not provide a means for controlling the ratio of specific carotenoids in a plant thereby producing plants that produce specific valuable carotenoids.

In U.S. Patent No. 5,684,238 to Ausich *et al* DNA segments from *Erwinia herbicola* encoding enzymes geranylgeryanyl pyrophosphate synthase, phytoene synthase, phytoene dehydrogenase-4H, lycopene cyclase, beta-carotene hydroxylase, and zeaxanthin glycosylase are disclosed. The abovementioned patent provides a means for producing zeaxanthin or glycosylated zeaxanthin in a culture containing a precursor and a host containing one or more said DNA segments or a transformed plant containing said beta-carotene hydroxylase. However, the U.S. Patent does not provide a means for controlling the ratio of other carotenoids in a plant thereby producing plants that produce other valuable carotenoids.

In U.S. Patent No. 5,744,341 to Cunningham, Jr. *et al* DNA segments from *Arabidopsis thaliana* encoding the eucaryote enzymes epsilon-cyclase and beta-hydroxylase, and DNA segments from *Arabidopsis thaliana* and bacterium *Haematococcus pluvialis* encoding the enzyme isopentyl pyrophosphate isomerase are disclosed. The U.S. Patent suggests uses for the disclosed DNA segments, however the patent does not provide a means for controlling the ratio of specific carotenoids in a plant species using DNA segments encoding various carotenoid biosynthesis enzymes from the same species thereby producing plants that produce other valuable carotenoids.

In U.S. Patent No. 5,750,865 to Bird *et al* DNA segments homologous to part or all of the clone pTOM from tomato is provided as a means to modify carotenoid biosynthesis in plants by promoting or inhibiting the synthesis of various carotenoids. The clone pTOM encodes an enzyme with a significant degree of homology to the crtB gene of *Rhodobacter capsulatus* which encodes phytoene synthase. The abovementioned invention is used to promote or inhibit the carotenoid biosynthetic pathway, but the invention does not provide a means for controlling the ratio of specific carotenoids in a plant.

Although the above techniques have been successful in providing enhanced levels of certain carotenoids in bacterial hosts when the appropriate carotenoid precursor is provided to the host, it would be preferable to utilize a higher plant species wherein technical maintenance procedures would be minimized and yield of specific carotenoids could be optimized. While U.S. Patents to Hauptmann *et al* and Ausich *et al* disclose uses in higher plants, the carotenoid enzymes disclosed are of bacterial origin which are structurally distinct from the carotenoid enzymes of eucaryote origin. It is well known in the art that an enzyme from a bacterium can be functionally similar to an enzyme from a eucaryote, however, the enzymes are rarely structurally related and in many cases the enzymes can possess different secondary functions that in the heterologous host can be undesirable. While U.S. Patents to Bird *et al* and Cunningham *et al* disclose several DNA segments encoding carotenoid biosynthesis enzymes, the proposed uses for said DNA segments are in heterologous hosts which in certain cases may result in undesirable side effects.

Therefore, there still remains a need for isolation of DNA sequences encoding other carotenoid biosynthetic enzymes from other higher plants. There also remains a need to manipulate the carotenoid biosynthetic pathway in plants to enhance production of specific carotenoid compounds. Finally, there remains a need for transformed plant species, wherein each variety of transformed plant species comprises a combination of DNA sequences derived from a plant which when in the transformed plant species affects the accumulation of specific carotenoid compounds.

SUMMARY OF THE INVENTION

The present invention provides a transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of beta-cyclase. The present invention also provides a transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of beta-hydroxylase. The present invention further provides a transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of epsilon-cyclase, further still, and a transgenic plant material containing an isolated DNA encoding a marigold IPP isomerase. The present invention further provides a transgenic plant material containing more than one isolated DNA encoding a marigold enzyme having catalytic activity of an enzyme selected

from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-cyclase, and isopentyl pyrophosphate (IPP) isomerase.

The present invention provides a transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of beta-cyclase which produces an RNA antisense to an mRNA encoding beta-cyclase. The present invention also provides a transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of beta-hydroxylase which produces an RNA antisense to an mRNA encoding beta-hydroxylase. The present invention further provides a transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of epsilon-cyclase which produces an RNA antisense to an mRNA encoding epsilon-cyclase. The present invention further provides a transgenic plant material containing more than one isolated DNA encoding a marigold enzyme having catalytic activity of an enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, and epsilon-cyclase wherein the RNA produced by the isolated DNA is antisense to an mRNA encoding an enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, and epsilon-cyclase.

The present invention further provides a transgenic plant material containing more than one isolated DNA encoding a marigold enzyme having catalytic activity of an enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-cyclase and epsilon-hydroxylase wherein the RNA produced by at least one of the isolated DNAs is antisense to an mRNA encoding an enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, and epsilon-cyclase.

Thus, the present invention provides genetically engineered marigold plants that over-produce a desired carotenoid pigment in the petal. The present invention further provides a method for transforming marigold plants with various combinations of isolated DNAs which encode at least one of the enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, IPP isomerase and epsilon-hydroxylase. The present invention allows the use of marigolds, a plant with known agronomic traits to produce a range of carotenoids in amounts that previously were not economically produced by traditional agricultural methods.

In the present invention, an isolated DNA encoding one or more of the enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, and beta-hydroxylase is operably linked to a promoter in the antisense orientation. The isolated DNA is introduced into the plant to make a transgenic plant. The isolated DNA in the plant is transcribed into an antisense RNA which is complementary to the mRNA transcribed from the corresponding carotenoid biosynthesis pathway gene in the plant's genome. The antisense RNA and the plant's mRNA form a double-stranded RNA duplex with the mRNA which inhibits translation of the mRNA, preventing synthesis of the enzyme. The isolated DNA can range in length from 50 nucleotides to the full length of the mRNA.

In another embodiment of the present invention an isolated DNA encoding one or more of the enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, and IPP isomerase is operably linked to a promoter in the sense orientation. The isolated DNA is introduced into the plant to make a transgenic plant. The isolated DNA in the plant is transcribed into an mRNA which is additive to the mRNA that is concurrently transcribed from the corresponding carotenoid biosynthesis pathway gene in the plant's genome. Thus an excess of mRNA encoding the desired carotenoid synthesis enzyme is produced. The excess mRNA is translated into the wanted enzyme producing an excess of the enzyme. Since there is now an excess of this enzyme, the excess enzyme out competes with other enzymes in the pathway for substrate. Thus, the carotenoid biosynthesis pathway is shifted towards the direction of those carotenoid products produced by the wanted enzyme.

In a third embodiment, a first isolated DNA encoding one or more of the enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, and epsilon-hydroxylase is operably linked to a promoter in the antisense orientation and a second DNA encoding one or more enzymes from the group not selected for antisense expression or IPP isomerase is operably linked to a promoter in the sense orientation. The isolated DNA is introduced into the plant to make a transgenic plant. The first DNA in the plant is transcribed into an antisense RNA which is complementary to the mRNA transcribed from the corresponding carotenoid biosynthesis pathway gene in the plant's genome. The second isolated DNA in the plant is transcribed into an mRNA which is additive to the mRNA transcribed from the corresponding carotenoid biosynthesis pathway gene in the plant's genome causing an excess of the enzyme to be produced. The

simultaneous inhibition of certain of these enzymes and overproduction of other of these enzymes causes the preferential accumulation of specific carotenoid products.

The preferred promoter to produce the anti-sense or the sense RNA is a promoter that specifically operates in the petals of the plant. Thus the carotenoid accumulates in the flower of the plant.

Transgenic plants containing the marigold genes regulated by the preferred petal-specific promoter allows the greatest level of production of the selected carotenoids in the petal of the transgenic plant to be achieved without affecting other tissues of the plant.

OBJECTS

It is an object of the present invention to provide isolated DNA sequences from marigold plants which encode enzymes involved in the carotenoid biosynthesis pathway. The isolated DNA sequences encode enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, and IPP isomerase. It is also an object to provide a petal specific promoter to produce RNA from the isolated DNA in the petal of the plant.

Another object of the present invention is to provide a method for producing a carotenoid in a marigold plant selected from the group consisting of beta-carotene, alpha-carotene, zeaxanthin, lycopene, zeinoxanthin, beta-cryptoxanthin, and combination thereof using the abovementioned isolated DNA sequences to produce RNA in the plant that are antisense to the mRNA concurrently produced by the plant. Therefore, a plant transformed with a vector that produces RNA antisense to epsilon-cyclase mRNA will cause the plant to preferentially accumulate zeaxanthin; a plant transformed with vectors that produce RNA antisense to epsilon-cyclase and beta-cyclase mRNAs will cause the plant to preferentially accumulate lycopene; a plant transformed with vectors that produce RNA antisense to epsilon-hydroxylase and beta-hydroxylase mRNAs will cause the plant to preferentially accumulate alpha-carotene; and a plant transformed with vectors that produce RNA antisense to epsilon-cyclase and beta-hydroxylase mRNAs will cause the plant to preferentially accumulate beta-carotene.

Another object of the present invention is to produce transgenic marigold which overproduce specific carotenoid biosynthesis enzymes which then causes the preferential accumulation of specific carotenoids in the petal. To accomplish the objective, the isolated DNA sequences are operably linked to a promoter in the sense orientation to produce a mRNA in the sense orientation. The present invention further provides for transformed marigold plants containing one or more of the isolated DNA sequences in the plant which causes an excess of each of the enzyme encoded by the isolated DNA to be made. The excess enzyme encoded by the isolated DNA affects the ratio of specific carotenoids in the transgenic plant, causing the over accumulation of specific carotenoids. The carotenoids to be overproduced are selected from the group consisting of beta-carotene, alpha-carotene, zeaxanthin, lycopene, zeinoxanthin, beta-cryptoxanthin, rubixanthin, and combination thereof.

Further still an object of the present invention is to provide transformed marigold plants containing various combinations of the isolated DNA sequences wherein certain DNA sequences are operably linked to a promoter which produce RNA in the sense orientation and other DNA sequences are operably linked to a promoter which produce RNA in the antisense orientation. The invention can be used to overproduce a carotenoid selected from the group consisting of beta-carotene, alpha-carotene, zeaxanthin, lycopene, zeinoxanthin, beta-cryptoxanthin, rubixanthin, and combination thereof.

These and other objects will become increasingly apparent by reference to the following description and the drawings.

DETAILED DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow diagram showing a part of the carotenoid pathway in higher plants.

Figure 2 is a flow diagram showing the reactions catalyzed by beta-cyclase and epsilon-cyclase.

Figure 3 is the DNA sequence for beta-cyclase (SEQ ID NO:1).

Figure 4 is the amino acid sequence for beta-cyclase (SEQ ID NO:2).

Figure 5 is the DNA sequence for epsilon-cyclase (SEQ ID NO:3).

Figure 6 is the amino acid sequence for epsilon-cyclase (SEQ ID NO:4).

Figure 7 is the DNA sequence for beta-hydroxylase (SEQ ID NO:5).

Figure 8 is the amino acid sequence for beta-hydroxylase (SEQ ID NO:6).

Figure 9 is the DNA sequence for isopentyl pyrophosphate (IPP) isomerase (SEQ ID NO:7).

Figure 10 is the amino acid sequence for IPP isomerase (SEQ ID NO:8).

DETAILED DESCRIPTION OF THE INVENTION

To facilitate the detailed description of the present invention, it is helpful to set forth definitions of certain terms to be used hereinafter.

Amino acids are the structural units comprising a polypeptide.

Nucleic acids are the structural units comprising a DNA or RNA molecule.

Transcription means the formation of an RNA chain in accordance with the genetic information contained in the DNA. When the genetic information encodes a structural gene, the RNA so formed is referred to as mRNA.

Translation means the process whereby genetic information in a mRNA molecule directs the order of specific amino acids during protein synthesis.

Expression means the combination of cellular processes, including transcription and translation undergone by a structural gene to produce a polypeptide.

Expression vector means a plasmid or phage comprising a DNA sequence operably linked to a promoter which in a cell causes transcription of the DNA into an RNA molecule. The RNA molecule can be translated into a polypeptide.

Operably linked means a DNA sequence linked to a promoter wherein the promoter causes the DNA sequence to be transcribed into an RNA molecule. The DNA sequence can comprise a structural gene, a portion of a structural gene, or a structural gene or portion thereof in the antisense orientation.

Promoter means a DNA sequence which causes transcription of DNA into a RNA molecule. For purposes herein, promoter is used to denote DNA sequences that permit transcription in a plant.

Recombinant DNA molecule means a hybrid DNA sequence comprising at least two nucleotide sequences not normally found together in nature.

Structural gene means a DNA sequence that is transcribed into an mRNA which is then translated into a polypeptide.

Vector means a DNA molecule that is capable of replicating in a cell and to which another DNA sequence can be operably linked so as to bring about replication of the attached DNA sequence. Commonly used vectors are bacterial plasmids and bacteriophages.

Sense refers to the sequence of the DNA strand of a structural gene that is transcribed into an mRNA molecule copy which is then translated into the polypeptide encoded by the structural gene.

Antisense refers to the sequence of the DNA strand that is complementary to the sequence of the sense strand and cannot be translated into the polypeptide encoded by the structural gene. For purposes of the present invention, antisense refers to a DNA that is operably linked to a promoter in the reverse orientation such that when the DNA is transcribed, an antisense RNA molecule is produced that has a nucleotide sequence that is complementary to and capable of hybridizing to an mRNA produced from the same DNA sequence in the sense orientation.

Polypeptide means the sequence of amino acids that comprise a structural gene. The term protein is equivalent to the term polypeptide. Enzymes are polypeptides.

Transformation means the process of introducing DNA into an organism which changes the genotype of the recipient organism in a stable manner. Transformation encompasses the introduction of the DNA by whatever means.

Transgenic plant means a plant which by the process of transformation is made to contain DNA sequences which are not normally present in the plant or DNA sequences which are in addition to the sequences which are normally present in the plant.

Polyadenylation site is the nucleotide sequence which causes certain enzymes to cleave mRNA at a specific site and to add a sequence of adenylic acid residues to the 3' end of the mRNA.

Marigold flowers have been used by the food and feed industries as a source of carotenoid pigments. The object of the present invention is to genetically engineer marigold plants to over-produce in the petals a desired carotenoid pigment. Marigold petals normally contain 1 to 3% zeaxanthin and greater than 90% lutein. Marigold plants that preferentially accumulate other carotenoids can be made according to the present invention. Marigold plants transformed with various combinations of isolated DNAs which encode at least one of the enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, and IPP isomerase. The transformed marigold plants are genetically engineered wherein certain genes of the carotenoid biosynthesis pathway (Figure 1) are either over-expressed or suppressed to deviate the carotenoid synthesis pathway in the desired direction which thus causes accumulation of desired carotenoids.

The carotenoids are preferably accumulated in the marigold flowers by using petal specific promoters operably linked to the abovementioned isolated DNAs. The petal-specific promoter allows the modification of carotenoid biosynthesis to be relegated to the petals of the transgenic plant. This allows carotenoid production to be manipulated without affecting or harming other tissues of the plant. Standard technology can be used to isolate the accumulated carotenoids from the flowers of the transformed marigolds. The present invention allows the use of marigolds, a plant with known agronomic traits to produce a

range of carotenoids and in amounts that previously were not economically produced by traditional agricultural methods.

Carotenoids are the most widespread group of pigments found in virtually all photosynthetic organisms and certain non-photosynthetic bacteria and fungi. In photosynthetic organisms, carotenoids are an essential component of the photosynthetic pathway. Glyceraldehyde-3-phosphate and pyruvate are used as substrates to produce dimethylaryl pyrophosphat (DMAPP) by a series of reactions known as the alternative IPP pathway. Many of the enzymes have yet to be described and cloned. DMAPP is converted to IPP and then to geryanylgeranyl pyrophosphate (GGPP) through an isomerization reaction catalyzed by IPP isomerase followed by a series of condensation reactions by GGPP synthase. GGPP is dimerized by phytoene synthase to form phytoene, the first C_{40} carotenoid.

The part of the carotenoid biosynthesis pathway in higher plants that proceeds from phytoene is shown in Figure 1. Phytoene is converted to the first pigment carotenoid, lycopene, through a series of dehydrogenation reactions catalyzed by one or more desaturases. Lycopene can serve as a precursor for a variety of other pigmented carotenoids.

Lycopene can be converted to beta-carotene through two sequential cyclization reactions catalyzed by beta-cyclase. Beta-cyclase cyclizes the termini of lycopene to form beta-rings. The reactions catalyzed by beta-cyclase or epsilon-cyclase are shown in Figure 2.

Beta-carotene can then be converted to zeaxanthin by two sequential hydroxylation reactions catalyzed by beta-hydroxylase which adds hydroxyl groups to the number 3 carbons of each beta-ring.

Lycopene can also be converted to alpha-carotene through two sequential cyclization reactions, the first reaction is catalyzed by epsilon-cyclase which forms the intermediate delta-carotene which has an epsilon-ring at one terminus and the second reaction, catalyzed by beta-cyclase, cyclizes the other terminus to form a beta-ring. The reactions are shown in Figure 1.

Alpha-carotene can be converted to alpha-cryptoxanthin in a reaction catalyzed by epsilon-hydroxylase which adds a hydroxyl group to the number three carbon of the epsilon-ring. A second hydroxylation reaction catalyzed by beta-hydroxylase converts alpha-cryptoxanthin to lutein by adding a hydroxyl group to the number three carbon of the beta-ring (Figure 1).

In addition to converting lycopene to beta-carotene, beta-cyclase can convert neurosporene to beta-zeacarotene which is then converted by a desaturase to gamma-carotene. Gamma-carotene can then be converted to beta-carotene by beta-cyclase or alpha-carotene by epsilon-cyclase. Neurosporene can also serve as a substrate for epsilon-cyclase which converts it into alpha-zeacarotene which is then converted to delta-carotene by a desaturase. Beta-cyclase can further convert delta-carotene to alpha-carotene.

Beta-hydroxylase can also convert alpha-carotene to zeinoxanthin which can then be converted to lutein in a reaction catalyzed by epsilon-hydroxylase.

The complexity of the pathway and the diversity of products formed in the reactions catalyzed by beta-cyclase, epsilon-cyclase, beta-hydroxylase, and epsilon-hydroxylase indicates that the pathway can be engineered to produce specific carotenoid products by altering expression of any one or several of the abovementioned enzymes.

Thus, the object of the present invention is to produce genetically engineered marigold plants which preferentially overproduce a desired carotenoid pigment in the petal. The present invention provides transgenic marigold plants which contain at least one of the isolated DNAs encoding the carotenoid biosynthesis gene selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, IPP isomerase, epsilon-hydroxylase, and combinations thereof to produce a transgenic marigold which preferentially accumulates in the petal a specific carotenoid biosynthesis pigment. The present invention provides isolated DNAs encoding beta-cyclase, epsilon-cyclase, beta-hydroxylase, and IPP isomerase from the marigold plant. The present invention also provides a method for transforming marigold plants with the isolated DNAs which encode at least one of the enzymes selected from the group consisting of beta-cyclase, epsilon-cyclase, beta-hydroxylase, IPP isomerase, epsilon-hydroxylase and combinations thereof

to produce a marigold plant which preferentially accumulates a specific carotenoid pigment in the petal.

Thus, the present invention provides an isolated DNA encoding beta-cyclase (Figure 3) wherein the isolated DNA has a sequence essentially the same as the sequence in SEQ ID NO.:1 wherein the sequence between positions 304 to 1836 encodes an enzyme having an amino acid sequence (Figure 4) essentially the same as the amino acid sequence in SEQ ID NO.:2. The isolated DNA of marigold encoding beta-cyclase was cloned in the plasmid pBSII KS+ (Stratagene, La Jolla, CA) which was deposited under the terms of the Budapest Treaty at the American Type Culture Collection (ATCC), 10801 University Blvd. Manassas, VA 20110-2209, USA on 28 July 1999 as ATCC PTA-447.

The present invention also provides an isolated DNA sequence encoding beta-hydroxylase wherein the isolated DNA has a sequence (Figure 7) essentially the same as the sequence in SEQ ID NO.:3 wherein the sequence between positions 51 to 923 encodes an enzyme having an amino acid sequence (Figure 8) essentially the same as the amino acid sequence in SEQ ID NO.:4. The isolated DNA of marigold encoding beta-hydroxylase was cloned in the plasmid pBSII KS+ which was deposited under the terms of the Budapest Treaty at the ATCC on 28 July as ATCC PTA-445.

The present invention further provides an isolated DNA sequence encoding epsilon-cyclase wherein the isolated DNA has a sequence (Figure 5) essentially the same as the sequence in SEQ ID NO.:5 wherein the sequence between positions 141 to 1688 encodes an enzyme having an amino acid sequence (Figure 6) essentially the same as the amino acid sequence in SEQ ID NO.:6. The isolated DNA of marigold encoding epsilon-cyclase was cloned in the plasmid pBSII KS+ which was deposited under the terms of the Budapest Treaty at the ATCC on 28 July as ATCC PTA-446.

The present invention further provides an isolated DNA sequence encoding IPP isomerase wherein the isolated DNA has a sequence (Figure 9) essentially the same as the sequence in SEQ ID NO.:7 wherein the sequence between positions 101 to 796 encodes an enzyme having an amino acid sequence (Figure 10) essentially the same as the amino acid sequence in SEQ ID NO.:8. The isolated DNA of marigold encoding IPP isomerase was cloned in the plasmid pBSII KS+ which was deposited under the terms of the Budapest Treaty at the ATCC on 28 July as ATCC PTA-448.

In a first embodiment of the present invention, a transgenic plant material is provided containing at least one isolated DNA encoding a marigold enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, and epsilon-cyclase wherein the isolated DNA is operably linked to a RNA promoter which in the plant produces an RNA that is antisense to the mRNA encoding the corresponding enzyme which is concurrently being produced by the plant. The isolated DNA, operably linked to a promoter to produce the antisense RNA, is selected from the group consisting of SEQ ID NO.:1, preferably the sequence between positions 1 to 1836, SEQ ID NO.:3, preferably the sequence between positions 1 to 923, and SEQ ID NO.:5, preferably the sequence between positions 1 to 1688. The isolated DNA can range from 50 nucleotides to a length which corresponds to the length of the mRNA. In the preferred embodiment, the isolated DNA is operably linked to a promoter which is specific for transcription in the petal.

The present invention thus provides a method for producing a plant that preferentially accumulates either zeaxanthin, lycopene, alpha-carotene, beta-carotene, zeinoxanthin, or alpha-cryptoxanthin. The method comprises producing a transformed plant that contains a sequence selected from the group consisting of SEQ ID NO.:1, preferably the sequence between positions 1 to 1836, SEQ ID NO.3, preferably the sequence between positions 1 to 923, SEQ ID NO.:5, preferably the sequence between positions 1 to 1688 and combinations thereof, wherein the sequence is operably linked to a RNA promoter in the orientation which will produce an antisense RNA. The transformed plant produces the antisense RNA which inhibits the complementary mRNA (or pre-mRNA) produced by the plant that encodes the targeted carotenoid biosynthesis enzyme by forming a double-stranded RNA complex with the mRNA. The double-stranded complex is preferentially degraded by enzymes in the plant which are specific for double-stranded RNA thereby reducing the amount of the targeted mRNA. Since the concentration of mRNA encoding the targeted enzyme is reduced or eliminated, the quantity of the targeted enzyme product is reduced or eliminated which causes the preferential accumulation of those carotenoids that are substrates for the enzyme that is targeted.

Thus, in the method of the present invention for producing a plant that preferentially accumulates zeaxanthin, the isolated DNA encoding epsilon-cyclase is operably linked to a promoter in the orientation that in the transgenic plant is transcribed into an antisense RNA. The antisense RNA binds the mRNA that encodes epsilon-cyclase which prevents

synthesis of the epsilon-cyclase enzyme. The inhibition of epsilon-cyclase synthesis causes a decrease in epsilon-cyclase in the plant which then causes the transformed plant to preferentially accumulate the carotenoid zeaxanthin.

In the method for producing a plant that preferentially accumulates lycopene, the transgenic plant contains the isolated DNA encoding epsilon-cyclase and the isolated DNA encoding beta-cyclase, operably linked to a promoter in the orientation which produces antisense RNA. The antisense RNAs bind the mRNAs encoding epsilon-cyclase and beta-cyclase, respectively, thereby preventing synthesis of the epsilon-cyclase and beta-cyclase enzymes. The decrease of the beta-cyclase and epsilon-cyclase enzymes causes the transformed plant to preferentially accumulate lycopene.

In the method for producing a plant that preferentially accumulates alpha-carotene, the transgenic plant contains the isolated DNA encoding epsilon-hydroxylase and the isolated DNA encoding beta-hydroxylase, operably linked to an promoter in the orientation which produces antisense RNA. The antisense RNAs bind to the complementary RNAs encoding epsilon-hydroxylase and beta-hydroxylase, respectively, preventing synthesis of epsilon-hydroxylase and beta-hydroxylase. The decrease of epsilon-hydroxylase and beta-hydroxylase causes the transformed plant to preferentially accumulate alpha-carotene.

In the method for producing a plant that preferentially accumulates beta-carotene, the transgenic plant contains the isolated DNA encoding epsilon-cyclase and the isolated DNA encoding beta-hydroxylase, operably linked to a promoter in the orientation which produces antisense RNA. The antisense RNAs bind their respective complementary mRNA which inhibits synthesis of the enzymes for beta-hydroxylase and epsilon-cyclase. The decrease of these enzymes causes the transformed plant to preferentially accumulate beta-carotene.

In the method for producing a plant that preferentially accumulates zeinoxanthin, the transgenic plant contains the isolated DNA encoding epsilon-hydroxylase, operably linked to a promoter in the orientation which produces antisense RNA. The antisense RNA binds the mRNA that encodes epsilon-hydroxylase which prevents synthesis of the epsilon-hydroxylase enzyme. The inhibition of epsilon-hydroxylase synthesis causes a decrease

of the epsilon-hydroxylase in the plant which then causes the transformed plant to preferentially accumulate the carotenoid zeinoxanthin.

In the method for producing a plant that preferentially accumulates alpha-cryptoxanthin, the transgenic plant contains the isolated DNA encoding beta-hydroxylase, operably linked to a promoter in the orientation which produces antisense RNA. The antisense RNA binds the mRNA that encodes beta-hydroxylase which prevents synthesis of the beta-hydroxylase enzyme. The inhibition of synthesis causes a decrease of the enzyme in the plant which then causes the transformed plant to preferentially accumulate the carotenoid alpha-cryptoxanthin.

In the aforementioned embodiments, the promoter that is operably linked to the isolated DNA to make the antisense RNA is a promoter that causes the transcription of the RNA from the isolated DNA to occur specifically in the petal of the marigold. An example of an RNA promoter that is specific for transcription in the petal is the Adonis ketolase promoter.

The present invention provides a transgenic plant material containing one or more isolated DNAs encoding marigold enzymes selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-hydroxylase, IPP isomerase and epsilon-cyclase wherein the beta-cyclase is encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 1836 in SEQ ID NO.:1, the beta-hydroxylase is encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 923 in SEQ ID NO.:3, the epsilon-cyclase is encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 1688 in SEQ ID NO.: 5, the epsilon-hydroxylase and the IPP isomerase is encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 796 in SEQ ID NO:7. The isolated DNA is operably linked to a promoter which in the host produces a functional mRNA that encodes the enzyme. In the preferred embodiment, the isolated DNA is operably linked to a promoter that is specific for transcription in the petal.

In another embodiment, the present invention provides a transgenic plant material containing combinations of isolated DNAs encoding marigold enzymes selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-hydroxylase, IPP isomerase and epsilon-cyclase wherein a first isolated DNA sequence is operably linked to a promoter to produce antisense RNA and a second isolated DNA sequence is operatively

linked to a promoter to produce an RNA that produces a functional enzyme. To produce the functional enzyme, the promoter is operably linked in the sense orientation to either beta-cyclase encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 1836 in SEQ ID NO.:1, the beta-hydroxylase encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 923 in SEQ ID NO.:3, the IPP isomerase encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 796 SEQ ID NO.:7, the epsilon-hydroxylase or the epsilon-cyclase encoded by the nucleotide sequence essentially homologous to the sequence between positions 1 to 1688 in SEQ ID NO.:5. To produce the antisense RNA, the isolated DNA is operably linked to the promoter in the antisense orientation and the length of the isolated DNA can range from 50 nucleotides to a length which corresponds to the full length of the mRNA. In the preferred embodiment, the isolated DNA is operably linked to a promoter that is specific for transcription in the petal.

Thus, the present invention provides a means for manipulating the carotenoid pathway in a plant to overproduce specific carotenoids and repress production of other carotenoids. For example, the present invention provides a means for inhibiting synthesis of epsilon-cyclase by introducing into the plant, DNA encoding RNA antisense to the epsilon-cyclase mRNA produced by the plant. Inhibition of epsilon-cyclase by the hybridization of the antisense RNA to the mRNA prevents synthesis of epsilon-cyclase which then reduces or prevents the conversion of neurosporene to alpha-zeacarotene, lycopene to delta-carotene, and gamma-carotene to alpha-carotene. Therefore, the carotenoid biosynthetic pathway will preferentially proceed towards the production of zeaxanthin. Inhibiting beta-hydroxylase in the same manner will prevent conversion of beta-carotene to zeaxanthin and zeinoxathin to lutein, thereby causing the accumulation of beta-carotene and zeinoxanthin.

In a second example according to the present invention, inhibition of the synthesis of the beta-cyclase and epsilon-cyclase enzymes is accomplished by introducing into the plant DNA encoding RNAs antisense to the beta-cyclase and epsilon-cyclase mRNAs produced by the plant. The antisense RNAs bind to their respective complementary mRNAs which inhibits translation of their respective mRNAs, thereby inhibiting synthesis of the beta-cyclase and epsilon-cyclase enzymes. The inhibition of the synthesis of the beta-cyclase and epsilon-cyclase enzymes reduces or eliminates conversion of neurosporene to beta-

zeacarotene and lycopene to beta-carotene and delta-carotene. Therefore, the primary product of the carotenoid in a pathway is lycopene.

In a third example according to the present invention, inhibition of synthesis of the beta-hydroxylase and epsilon-hydroxylase enzymes will cause the preferential accumulation of alpha-carotene. It also follows from this example that inhibition of beta-hydroxylase according to the present invention prevents alpha-cryptoxanthin from being converted to lutein, thereby causing accumulation of alpha-cryptoxanthin, and that inhibition of only epsilon-hydroxylase prevents zeinoxanthin from being converted to lutein, thereby causing accumulation of zeinoxanthin.

The present invention also provides for manipulation of the carotenoid biosynthesis pathway wherein any one of the abovementioned enzymes is overproduced in the plant. For example, overproduction of beta-cyclase according to the present invention will produce an excess of beta-cyclase which will more effectively compete with epsilon-cyclase for neurosporene and lycopene substrates thereby causing the carotenoid biosynthesis pathway to preferentially increase production of beta-carotene and zeaxanthin, and decrease production of alpha-carotene and its derivatives. Conversely, overproduction of epsilon-cyclase will cause the carotenoid biosynthesis pathway to shift towards production of alpha-carotene and its derivatives. Therefore, the present invention encompasses manipulation of the carotenoid biosynthesis pathway by providing to the plant, an isolated DNA containing at least one of the enzymes selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-cyclase and epsilon-hydroxylase which when transcribed into mRNA and translated in the plant, provides an additional amount of the carotenoid biosynthesis enzymes selected to be overproduced.

The genes encoding beta-cyclase, epsilon cyclase and beta-hydroxylase were isolated from marigold and cloned into a bacterial plasmid. The DNA sequence for beta-cyclase is shown in Figure 3. The gene encoding the beta-cyclase is 1533 bp and corresponds to nucleotide position 304 to 1836. The amino acid sequence for beta-cyclase is shown in Figure 4. The DNA sequence for epsilon-cyclase is shown in Figure 5. The gene encoding the epsilon-cyclase is 1548 bp and corresponds to nucleotide position 141 to 1688. The amino acid sequence for epsilon-cyclase is shown in Figure 6. The DNA sequence for beta-hydroxylase is shown in Figure 7. The gene encoding the beta-hydroxylase is 873 bp and corresponds to nucleotide position 51 to 923. The amino acid sequence for beta-

cyclase is shown in Figure 8. The DNA sequence for IPP isomerase is shown in Figure 9. The gene encoding for IPP isomerase is 796 bp and corresponds to nucleotide positions 101 to 796. The amino acid sequence for IPP isomerase is shown in Figure 10. The petal specific promoter was isolated from *Adonis vernalis* and is the promoter regulating the ketolase gene. The marigold genes encoding geranylgeranyl pyrophosphate synthase and zeta-carotene desaturase have been cloned and sequenced.

Construction of clones containing the carotenoid biosynthesis DNA operably linked to a promoter can be accomplished using techniques well known in the art (for example Sambrook et al (1989)). Suitable vectors for eukaryote expression in plants are described in Frey *et al* (1995), and Misawa *et al* (1994), which are incorporated herein by reference.

Transgenic plants are constructed which contain the DNA sequences comprising the present invention. The incorporation of these sequences into the plant allows the carotenoid biosynthetic pathway to be manipulated to produce specific carotenoids. The manipulation can be by antisense inhibition, overproduction of selected carotenoid biosynthesis enzymes, or a combination thereof.

There are many methods known in the art for transforming a plant cell. Common methods include transformation with T-DNA containing the DNA of interest and using *A. tumefaciens* as the means for transformation or with Ti or Ri plasmids using the bacterium *A. rhizogenes* as the means for transformation. A suitable plasmid for transformations is the pART27/7 plasmid vector isolated from *Agrobacterium tumefaciens*. Other methods for transforming a plant cell include cell fusion, electroporation, biolistic or conventional injection.

Agrobacterium related methods require special plasmid vectors such as intermediate or binary vectors. Intermediate vectors require integration into Ti or Ri plasmids by homologous recombination into the region containing the T-DNA. The intermediate vector is transferred into the *Agrobacterium* by means of conjugation in the presence of a helper plasmid. The transformed *Agrobacterium* is then used to transform the cell. The preferred method for transforming *Agrobacterium* is using plasmids of the binary type. Binary vectors replicate both in *Escherichia coli* and *Agrobacterium*. Therefore, these vectors containing the desired DNA can be constructed using conventional molecular biology techniques and the recombinant plasmid directly transferred to *Agrobacterium*. Binary

vectors usually contain a marker gene and a polylinker for inserting the desired DNA flanked by the left and right T-DNA border regions. Both the intermediate and binary vectors contain the *vir* region which is necessary for transfer of the T-DNA into the plant cell.

Transformation of plant cells with transformed *Agrobacterium* is by co-cultivation of the cells with the transformed *Agrobacterium* which results in transfer of the T-DNA containing the desired DNA into the plant cell. Sources for plant cells are explants which can include but is not limited to sections of leaves, stems, roots, segments of petioles, flowers and flower parts, and cotyledon tissue. Whole plants are regenerated from the infected plant material or from protoplasts or suspension-cultivated cells in a suitable medium which can contain antibiotics or biocides (e.g., kanamycin, bleomycin, hygromycin, chloramphenicol) for selection of the transformed plant cells. The ability and efficiency of regenerating a transformed or transgenic plant using transformed isolated cells or explants is dependent on the species of plant and the type of transformed cell. Transformation of marigold tissue can be achieved according to the *Agrobacterium*-mediated method for transforming plants disclosed in U.S. Patent Nos. 5,684,238 to Ausich *et al* and 5,618,988 to Hauptmann *et al* which are herein incorporated by reference.

Non-*Agrobacterium* mediated transformation such as electroporation, injection, cell fusion, or particle bombardment do not require special plasmids and can therefore use standard plasmids such as the pUC derivatives and conventional cloning techniques. For example, to make the transgenic marigold plants of the present invention using the Biolistic bombardment method, marigold tissue is transformed using the Biolistic method described in U.S. Patent No. 5,767,368 to Zhong *et al* which is herein incorporated by reference. Further examples of the Biolistic bombardment method are disclosed U. S. Patent No. 5,736,369 to Bowen *et al* which is herein incorporated by reference.

Expression of cloned DNAs such as the isolated DNAs of the present invention in the plant cell requires the isolated DNA to be operably linked to a promoter. The preferred promoter is the petal specific promoter from the ketolase gene of *Adonis vernalis* (pheasant's eye). Examples of other promoters which are useful are viral promoters such as the cauliflower mosaic virus 35S promoter, heat shock protein promoters such as the HSP70 promoter, light induced promoters such as the ST-Ls1 or the rubisco small subunit

promoter, stress response promoters such as the PR promoter, the *Agrobacterium tumefaciens nos* promoter, and various organ, root, tuber, leaf, and other flower specific promoters. Examples of other promoters contemplated are differentially regulated promoters which are promoters that operate in only certain plant tissues, under certain environmental conditions or at a particular developmental stages of the plant. The CRB promoter isolated from the CRB gene of the 12S seed protein of *Arabidopsis thaliana* which targets expression to the seed is one such differentially regulated promoter. The DRE promoter element that is inducible under stress is an example of a plant promoter that responds to environmental conditions (Yamaguchi-Shinozaki et al, 1994). The isolated DNA also requires being operably linked to a transcription termination signal. The termination signal can be the sequence naturally associated with the isolated DNA or can be a sequence operably linked to the 3' end of the isolated DNA. An example of such a sequence is the transcription termination signal of the octopine synthase gene.

In the embodiments of the invention wherein antisense RNA production is desired, the transcription of the isolated DNAs in the plant cell produces an RNA that is antisense to the mRNA or pre-mRNA of the gene product targeted for inhibition. James (1991) has reviewed antisense RNA and its use in gene inhibition therapy. Other reviews of antisense technology specifically directed to transgenic plants are by Senior (1998) and Nellen *et al.* (1996). Generally, the inhibition is affected in the cell nucleus by the formation of a double-stranded RNA consisting of one molecule of antisense RNA and one molecule of the mRNA forming a double helix molecule. The double helix molecule is preferentially degraded in the nucleus by enzymes that specifically degrade double-stranded RNA molecules. In this manner, the pool of mRNA available for translation is reduced or eliminated which in turns reduces the pool of enzyme encoded by the mRNA. The length of the antisense RNA that is effective for inhibition is between 50 nucleotides and a size which corresponds to the full length of the mRNA it is complementary with. The degree of inhibition affected by the present invention is at least 70% such as at least 80% including 90% preferably at least 98%, depending on the length of the antisense RNA and the particular region of the mRNA it is directed to when the antisense RNA is shorter than the mRNA. Thus, the present invention provides a method for substantially inhibiting a particular enzyme by using an RNA that is antisense to the enzymes mRNA.

The present invention describes transgenic marigold plants wherein the carotenoid biosynthesis pathway is manipulated to produce specific carotenoids by transforming

marigold tissue with various combinations of one or more isolated DNAs containing beta-cyclase, epsilon-cyclase, beta-hydroxylase, IPP isomerase or epsilon-hydroxylase in either antisense or sense orientation. However, manipulation of the carotenoid pathway according to the present invention can include other enzymes that are involved in the biosynthesis of carotenoids. These enzymes can be of marigold origin or from other organisms. Examples of such genes are the 1-deoxy-D-xylulose 5-phosphate synthase (DXP synthase) from *E. coli* (GenBank Accession No. U82664), the marigold homolog to the DXP synthase which produces a deep red when in the presence of lycopene, and the *Arabidopsis thaliana* homolog to DXP synthase (Cla 1 gene - GenBank Accession No. U27099). Thus, the present invention is not limited to the specific genes mentioned herein but also includes other genes encoding enzymes that are involved in carotenoid biosynthesis.

The following examples are intended to promote a further understanding of the present invention.

EXAMPLE 1

HPLC characterization of selected marigold lines including known color variants was performed to identify marigold color variants that had mutations in the carotenoid biosynthetic pathway. These mutations were expected to accumulate intermediates such as beta or alpha carotene or mono-hydroxy derivatives.

In normal orange marigold lines between 90 and 98% of their total carotenoid content is lutein. The vast majority of the lutein is esterified to fatty acids. HPLC analysis was performed on all commercially available marigold color variants such as the dark orange, red fringed, yellow, cream, and white variants among others. All the commercially available variants were identified as quantitative mutants, that is these variants accumulated less of each intermediate in the same proportion. In other words, none of the variants accumulated any intermediate at appreciable levels. Therefore, marigold variants that have useful carotenoid mutations that cause accumulation of carotenoid biosynthetic pathway intermediates appeared to be distant.

EXAMPLE 2

A cDNA library was constructed to screen for and isolate cDNAs encoding enzymes involved in the carotenoid biosynthetic pathway. To facilitate construction of the cDNA library, the mRNA levels for carotenoid biosynthetic steps during marigold flower development was analyzed to identify the appropriate stage of development to prepare the cDNA library. The cDNAs targeted were cDNAs encoding beta-cyclase, epsilon-cyclase, beta-hydroxylase, IPP isomerase and epsilon hydroxylase. It was also discovered that the corresponding cDNAs encoding beta-cyclase, epsilon-cyclase, and beta-hydroxylase from *Arabidopsis thaliana* hybridized to the corresponding marigold genes. This discovery enabled expression of the abovementioned carotenoid pathway mRNAs be directly evaluated during floral development.

Based on the analysis of mRNA levels, three of six arbitrary marigold floral development stages were selected for sources of RNA for library construction. A cDNA library containing more than 10^7 independent cDNAs was constructed and screened for cDNAs encoding beta-cyclase, epsilon-cyclase, beta-hydroxylase, and epsilon-hydroxylase. Briefly, poly(A+) RNA was isolated from developing marigold flowers and made into cDNA using art known methods. A cDNA library was made by Stratagene (La Jolla, California) using the Stratagene Lambda ZAP Cloning System. The library was non-directional in the vector and consisted of more than 10^7 independent clones. Various screening procedures were used including heterologous screening using relevant *Arabidopsis* genes, functional screening based on colour complementation and novel methods based on accelerated growth at low temperature. Identification of clones containing carotenoid biosynthesis enzymes was as follows.

Marigold beta-cyclase was identified by colour complementation of a lycopene accumulating *E. Coli* strain. This method is described in U.S. Patent No. 5,744,341 to Cunningham, Jr. *et al* which is herein incorporated by reference. Approximately 360,000 colonies were screened. Of these colonies, 4 yellow colonies were picked, and DNA was extracted from two of the colonies and the DNA sequenced. Figure 3 shows the DNA sequence for the marigold beta-cyclase. The amino acid sequence for beta-cyclase was deduced from the DNA sequence and is shown in Figure 4.

Marigold epsilon-cyclase was identified by plaque hybridizations using as the probe the *Arabidopsis thaliana* epsilon-cyclase (GenBank Accession No: U50738). A DNA clone containing an epsilon cyclase from *Arabidopsis thaliana* that is suitable for use as a probe to screen the library is available from the ATCC as ATCC-98005. Approximately 280,000 plaques were screened and 9 plaques were purified. DNA was isolated from 2 of the plaques and the DNA was sequenced. The DNA sequence is shown in Figure 5. The amino acid sequence for epsilon-cyclase was deduced from the DNA sequence and is shown in Figure 6.

Marigold beta-hydroxylase was identified by plaque hybridizations using as the probe the *Arabidopsis* beta-hydroxylase. A DNA clone suitable for use as a probe to screen the library is available from the ATCC as ATCC-98003. Approximately 280,000 plaques were screened and 13 plaques were purified. DNA was isolated from 3 plaques and the DNA was sequenced. The DNA sequence is shown in Figure 7. The amino acid sequence for beta-hydroxylase was deduced from the DNA sequence and is shown in Figure 8.

Marigold IPP isomerase was identified by using a cold screen method in which zeaxanthin expressing *E. coli* were transformed with the marigold cDNA library and grown at 18°C. Rapidly growing pigmented colonies which contained the IPP isomerase were characterized. Five independent colonies were further shown to contain marigold IPP isomerases. Four of these clones were partially sequenced and one of these clones was fully sequenced. All of these clones were closely related but not identical. The DNA sequence is shown in Figure 9. The amino acid sequence for IPP isomerase was deduced from the DNA sequence and is shown in Figure 10.

Clones from a marigold cDNA library encoding geranylgeranyl pyrophosphate synthase and zeta-carotene desaturase have been identified by homology to their homologous genes in *Arabidopsis*. These genes have been isolated and sequenced.

EXAMPLE 3

Regeneration of marigold plants is a key element for successful generation of transgenic plants, however there is little information regarding tissue culture of marigold. Therefore, the objective of this example was to develop a method for the regeneration of marigold *in*

vitro. As part of our objective, several commercial and proprietary marigold genotypes were evaluated for germination and growth in culture.

Identification of marigold genotypes that regenerated best *in vitro* was performed by evaluating the number of adventitious shoots per experiment. All varieties of marigold plant tissue were evaluated.

Table 1: Summary of the morphological responses of marigold tissues to various hormonal concentrations and combinations.

MEDIA		TISSUE RESPONSE
BA (1.0 mg/l)	IAA (1.0 mg/l)	R-
	IAA (3.0 mg/l)	R-S-
	IAA (5.0 mg/l)	C+
BA (3.0 mg/l)	IAA (1.0 mg/l)	S++, R-, C+
	IAA (3.0 mg/l)	S++
	IAA (5.0 mg/l)	S+, C+
BA (5.0 mg/l)	IAA (1.0 mg/l)	S++, C+
	IAA (3.0 mg/l)	S+, C+
	IAA (5.0 mg/l)	S-, R+, C+
BA (1.0 mg/l)	IAA (0.5 mg/l)	S+, C++
BA (5.0 mg/l)	IAA (3.0 mg/l	S+++, R+, C++

R= roots, S= shoots, C= callus
+++ = excellent development;++ = very good development; + = good development; - = poor development

Regeneration potential of marigold was evaluated by monitoring the morphological response of marigold tissues to various hormonal concentrations and combinations. Regeneration was evaluated in three stages: shoot induction, shoot elongation, and rooting. The first stage, shoot induction, was performed as follows. The media was Murashige and Skoog (MS) medium containing various concentration of benzyladenine (BA) ranging from 1.0 mg/l to 5.0 mg/l. At each concentration of BA, various concentrations of IAA were added, ranging from 0.5 mg/l to 5.0 mg/l. Table 1 shows that MS media containing 5.0 mg/l BA and 3.0 mg/l IAA was the best medium for regenerating transformed marigold cultures.

Shoot buds, differentiated as above, are subcultured in the same media as above every two weeks for multiplication of shoots, as long as the regeneration from callus continues. Once shoots are visible from callus or original explants they are subcultured to MS media containing one tenth of the hormones used for shoot induction.

In the next stage, shoot elongation, shoot buds from above are subcultured on MS media without BA and IAA. In the final stage, rooting, the tissue from the shoot elongation stage is further subcultured in media without BA and IAA.

Table 2 summarizes the response of different marigold explants to media containing different hormones.

Table 2: Summary of the response of different marigold explants to media containing different hormones.

MARIGOLD VARIETY	MORPHOGENIC RESPONSE	STATUS
CLIMAX HYBRID TOREADOR (1)	S+, R+, C+, NGR+	
GOLDEN CLIMAX HYBRID	S++, R++, C+, NGR++	
XANTHOPHYLL SCARLETADE	S+, C++	
XANTHOPHYLL ORANGEADE	S+, C++	
XANTHOPHYLL DEEP ORANGE	S+, C++	
O32-442 (5287)	S-, C++, NGR++	leaf, stem, cotyledon
032-439 (1273)	S++, R++, C++, NGR-	leaf, stem, cotyledon
36969	S++, R++, C++, NGR-	cotyledon
36898	S++, R++, C++, NGR-	cotyledon
032-440 (1274)	S+, R-, C++, NGR-	cotyledon

R= roots, S= shoots, C= callus, NGR= negative geotropic roots
+++ = excellent development; ++ = very good development; + = good development; - = poor development

There were recurring problems with most genotypes which was manifested as browning of the tissue and growth of non-geotropic roots (growth of roots can be a problem during regeneration, because once the roots start to form, the growth of other plant structures decreases). However, in terms of regeneration of marigold plants from untransformed tissue, many plants have been regenerated from different explants, tissues and genotypes according to the method shown herein.

EXAMPLE 4

Initial marigold tissue culture transformations were performed to determine the appropriate tissue for transformation with *Agrobacterium* and then plant regeneration from transformed tissue. The *Agrobacterium* that was used was *Agrobacterium* LB4404 containing in most cases the transformation vector pBI121 which contained the CaMV 35S promoter driving the beta-glucuronidase reporter gene (GUS) and the NPTII gene as the selectable marker. The beta-glucuronidase cleaves the colorless substrate, X-glu, producing a product having a blue color.

Leaves were selected as the tissue from marigold for transformation because leaves are generally an easy regenerating tissue providing healthy plants back from leaves in culture after approximately eight to six weeks. Large-scale transformations were initiated in earnest using six Pan American marigold lines in case there were cultivar variations as in tomato which would affect transformation. Over 5,000 independent leaf sections were individually transformed by *Agrobacterium*-mediated transformation and carried through regeneration attempts for approximately eight to twelve weeks, with weekly or bi-weekly transfers for each transformation event. Despite the number of transformations not a single transformation event scored as transformed plantlets were ever identified even though transformed callus tissue that proliferated roots could be obtained. However, the transformed callus tissue was recalcitrant to plant regeneration.

It was observed that during these transformation attempts, many of the transformed tissues turned brown, would not show any response to hormones, and eventually died. Several alternative approaches were tried to transform marigold leaf tissue. Among them being using different tissues for transformation, and using other strains of *Agrobacteria* as the transforming agent. Because marigolds produce thiophenes which are natural antibacterial compounds and may inhibit Agrobacterium-mediated transformation,

transformants were co-cultivated in the dark (light activates thiophenes), transformations were performed with low thiophene producing strains of marigolds, or transformants were co-cultivated in sulfate deficient media (to decrease thiophene production *in vitro*). None of these variations produced transformed plants. Therefore, the conclusion was that despite the ability of marigold leaf tissue to regenerate better than any other plant tissue, marigold leaves were difficult to transform (less than 1% efficiency) and the tissue that was transformed could not be made to regenerate into plants.

Since marigold leaves were refractory to regeneration after transformation, other marigold tissue was evaluated for regeneration and transformation. Marigold cotyledon tissue was tested for ability to be transformed. Cotyledon tissue was transformed with *Agrobacterium* LB4404. Transformed cotyledon tissue is capable of transformation several independent transformation events have produced transformed plants capable of growth in soil.

The protocol for marigold transformation that was developed using *Agrobacterium* is set forth below.

Induction and inoculation. Two weeks prior to the experiment, germinate seeds aseptically in MS media and agar plates. Two days prior to inoculation, cut off cotyledons from seedlings and place them on MS media containing hormones as described in Example 3, and incubate under standard conditions. One to two days prior to inoculation, streak *A. tumefaciens* onto a petri plate containing LB agar and grow for two days with appropriate antibiotics.

On day of inoculation, scrape the new growth bacteria from the culture plate and make a mixture using induction medium in MS media. Shake the mixture for 30 minutes before using. Using sterile forceps transfer all cotyledons to a plate and then add the bacteria mixture and vacuum infiltrate for 5 minutes. Then remove all explants from the bacteria mixture and place the bacteria coated explants back into the same media they had been growing in for co-cultivation. The co-cultivation period allows the bacteria and plant material to remain in close proximity for 2 to 3 days. After the co-cultivation period, transformed plant tissue is selected by transferring all the explants to the same media containing antibiotics to kill the *Agrobacterium* and kanamycin or hygromycin to select for transformants. Transformants can also be selected for herbicide resistance, provided that the transformed tissue is cotransformed with DNA encoding a herbicide resistance gene

and the selection is performed in media containing the herbicide. Regeneration is essentially as described in Example 3.

The following three transformation experiments were done using the 35S-GUS-HYG construct (similar to pBI121 except encoding resistance to the antibiotic hygromycin) in LB4404. The explant used was cotyledons from aseptically grown marigold seedlings for each genotype. Regeneration was essentially as described in Example 3. The results shown in Table 3, demonstrate that using the transformation conditions and regeneration conditions described herein, cotyledon tissue from marigolds can be transformed and regenerated into plants. This important discovery provides both the method and transgenic marigold of the present invention.

Table 3: Summary of transformation experiment using the 35S-GUS-HYG construct.

Variety	No. Explants inoculated	Transformants GUS positive	Total plantlet clones (after subculture)
032-439 (1273	320	6	48
36969	300	5	28
36969	350	4	25

The use of cotyledons allowed plantlets to be regenerated following inoculation with *Agrobacterium*. Even though the transformation efficiency of cotyledon was not much better than the efficiency for transforming leaves, the transformed cotyledon tissue is capable of being regenerated into plants. Currently, there are two transformed plants from transformed 032-439 in the soil. There is one transformed 36969 plant in the soil with several others ready for planting. Thus, the method developed herein will produce transgenic marigold plants from transformed cotyledon tissue.

EXAMPLE 5

As an alternative to *Agrobacterium* mediated transformation, each transformation of the nuclear genome of marigold is accomplished by transforming marigold tissue such as cotyledon tissue or shoot-tips with one of the three isolated DNAs. The DNAs are coprecipitated onto 1.0 μm tungsten particles according to the method described by

U.S. Patent No. 5,320,961 to Zhong et al. Multiple marigold shoot-tip clumps are initiated from shoot tips of marigold seedlings and maintained in light for 4-week intervals on Murashige and Skoog (MS) medium containing 2 mg/ml benzyladenine (BA) and 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Shoot tips and shoot clumps are physically exposed by removal of the leaves, when necessary, and placed in a circular area having a diameter of approximately 1.5 cm prior to transformation. Alternatively, cotyledon tissue can be transformed by Biolistic bombardment.

Transformation consists of bombarding the shoot tips and clumps with the tungsten particles coated with the DNA precipitate using a Biolistic particle acceleration device (PDS 1000/He, Bio-Rad, Hercules, CA USA) under a chamber pressure of 26 mm of Hg at distances of 1.5, 2.0 and 6.5 cm from the rupture disc to the macrocarrier to the stopping screen to the target, respectively, with a density of 150 µg/shot of the coated tungsten particles with 4 shots and 1,550 p.s.i. acceleration pressure.

Afterwards, the bombarded tissue is cultured on MS medium containing 2 mg/ml BA and 0.5 mg/L 2,4-D for 6 to 8 weeks. This important step is necessary to reduce the degree of chimerism in the transformed tissue. Afterwards, the green clumps are selected, divided and subcultured in the above medium. Then, those plantlets that have normal root development are transferred to pots and acclimated to soil conditions before being transferred to greenhouses.

Production of specific carotenoid compounds is determined using methods described in Example 1. In addition, a selection method such as antibiotic resistance (Example 5) or herbicide resistance can be incorporated into this method by co-transforming the plant tissue an isolated DNA that encodes for antibiotic resistance or herbicide resistance and cultivating the transformed tissue in the presence of the antibiotic or herbicide.

EXAMPLE 6

To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence from the beta-cyclase gene as shown in SEQ ID NO:1, marigold cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the antisense orientation.

EXAMPLE 7

To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence from the beta-hydroxylase gene as shown in SEQ ID NO:3, marigold cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the antisense orientation.

EXAMPLE 8

To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence encoding the epsilon-cyclase gene as shown in SEQ ID NO:5, marigold cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the antisense orientation.

EXAMPLE 9

To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence from the beta-cyclase gene as shown in SEQ ID NO:1, marigold cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the sense orientation which encodes beta-cyclase.

EXAMPLE 10

To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence from the beta-hydroxylase gene as shown in SEQ ID NO:3, marigold cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the sense orientation which encodes beta-hydroxylase.

EXAMPLE 11

To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence encoding the epsilon-cyclase gene as shown in SEQ ID NO:5, marigold

cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the sense orientation which encodes beta-cyclase.

EXAMPLE 12

To make a transgenic marigold plant containing an isolated DNA that contains a DNA sequence from the IPP isomerase gene as shown in SEQ ID NO:7, marigold cotyledon tissue is transformed as in Example 4 or 5. The transformed tissue is used to make the transgenic plant. The DNA sequence produces RNA in the sense orientation which encodes IPP isomerase.

EXAMPLE 13

Transgenic marigold plants containing more than one isolated DNA containing a carotenoid biosynthesis synthesis gene in either the antisense or the sense orientation is made by cross-breeding the transgenic plants (made according to Examples 6 to 12) which contain isolated DNA containing the sequence from SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO 7, according to methods well known in the art such as those provided in (Zhang et al, 1996; Zhong et al, 1996; Zhong et al,1992). Transgenic plants that carry a low copy number of the isolated DNA used for cross-breeding.

Briefly, transgenic marigold plants that contain more than one isolated DNA are made by first making transgenic plants that contain either SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or SEQ ID NO:7 to make a first, a second, a third and a fourth transgenic plant. The first and second transgenic plants are cross-bred to create a bi-transgenic plant (contains SEQ ID NO:1 and SEQ ID NO:3) which can then cross-bred with the third transgenic plant to make a tri-transgenic plant which contains isolated DNAs containing SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5. The fourth transgenic plant can be crossed with the tri-transgenic plant to produce the quadri-transgenic plant containing SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 and SEQ ID NO:7. In the above-described manner, transgenic plants containing any combination and any number of isolated DNAs can be constructed.

Transgenic plant lines containing more than one isolated DNA are cross-pollinated with transgenic plant lines containing another isolated DNA. The resulting hybrid progeny are

cross-pollinated with transgenic plant lines containing other isolated DNAs. Each transgenic plant line produces specific carotenoid compounds depending on both what isolated DNAs are contained by the plant and whether the DNAs express RNA in the antisense orientation, the sense orientation or a combination thereof.

Alternatively, transgenic plants containing more than one type of isolated DNA can be made by multiple transformations. For example, cotyledon tissue from a transgenic plant containing one of the isolated DNAs can be transformed with another of the isolated DNAs to produce the bi-transgenic plant as shown in Examples 4 to 5.

Another alternative for making transgenic plants containing more than one type of isolated DNA is to either simultaneously transform the cotyledon tissue with multiple isolated DNAs containing the desired gene sequences or transform with one isolated DNA that contains each desired gene sequence. Transformation can be as shown as in Examples 4 to 5.

EXAMPLE 14

Transgenic marigold plants containing an isolated DNA which contains more that one DNA sequence that produces antisense RNA to mRNA encoding at least two of beta-cyclase, beta-hydroxylase, or epsilon-cyclase are produced by a single transformation as shown in Example 4 or 5. The isolated DNA in this example contains DNA sequences from a combination of at least two DNA sequences selected from the group of DNA sequences which encodes beta-cyclase, beta-hydroxylase, or epsilon-cyclase wherein the DNA sequences are in the antisense orientation.

EXAMPLE 15

Transgenic marigold plants containing an isolated DNA which contains more that one DNA sequence that produces sense RNA encoding at least two of beta-cyclase, beta-hydroxylase, IPP isomerase or epsilon-cyclase are produced by a single transformation as shown in Example 4 or 5. The isolated DNA in this example contains DNA sequences from a combination of at least two DNA sequences selected from the group of DNA sequences which encodes beta-cyclase, beta-hydroxylase, IPP isomerase or epsilon-cyclase.

EXAMPLE 16

Transgenic marigold plants containing an isolated DNA which contains at least one DNA sequence that produces sense RNA encoding at least one of beta-cyclase, beta-hydroxylase, IPP isomerase, or epsilon-cyclase and at least one DNA sequence which produces antisense RNA to mRNA encoding at least one of beta-cyclase, beta-hydroxylase or epsilon-cyclase are produced by a single transformation as shown in Example 4 or 5. The isolated DNA in this example contains (1) a DNA sequence from at least one DNA sequence selected from the group of DNA sequences which encodes beta-cyclase, beta-hydroxylase, or epsilon-cyclase, and (2) a DNA sequence in the antisense orientation from at least one DNA sequence not selected in (1).

While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary skill in the art and access to the teachings herein will recognise additional modifications and embodiments within the scope thereof.

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Applicant's or agent's file reference number 23166 PC 1	International application No.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 15, line 11.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

10801 University Blvd.
Manassa, Va 20110-2209
USA

Date of deposit	Accession Number
28 July 1999	PTA-447

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
[ ] This sheet was received with the international application	[ ] This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO DEPOSITED MICROORGANISMS
(PCT Rule 12bis)

Additional sheet

In addition to the microorganism indicated on page 33 of the description, the following microorganisms have been deposited with

American Type Culture Colllection, 10801 University Blvd., Manassas, VA 20110-2209, USA.

on the dates and under the accession numbers as stated below:

Accession number	Date of deposit	Description Page No.	Description Line No.
PTA-445	28 July 1999	15	19
PTA-446	28 July 1999	15	27
PTA-448	28 July 1999	15	35

For all of the above-identified deposited microorganisms, the following additional indications apply:

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms stated above only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

CLAIMS:

1. A transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of beta-cyclase.

2. The transgenic plant material according to claim 1 wherein the isolated DNA encodes a marigold enzyme having catalytic activity of an enzyme encoded by SEQ ID NO.:1.

3. The transgenic plant material according to claim 2 wherein the isolated DNA encodes the enzyme from positions 304 to 1836 in SEQ ID. NO.:1.

4. The transgenic plant material according to claim 1 wherein the isolated DNA encodes a marigold enzyme having the amino acid sequence of SEQ ID NO.:2.

5. A transgenic plant material containing isolated DNA encoding a marigold enzyme having catalytic activity of beta-hydroxylase.

6. The transgenic plant material according to claim 5 wherein the isolated DNA encodes a marigold enzyme having catalytic activity of an enzyme encoded by SEQ ID NO.:3.

7. The transgenic plant material according to claim 6 wherein the isolated DNA encodes the enzyme from positions 51 to 923 in SEQ ID. NO.:3.

8. The transgenic plant material according to claim 5 wherein the isolated DNA encodes a Marigold enzyme having the amino acid sequence of SEQ ID NO.:4.

9. A transgenic plant material comprising isolated DNA encoding a marigold enzyme having catalytic activity of epsilon-cyclase.

10. The transgenic plant material according to claim 9 wherein the isolated DNA encodes a marigold enzyme having catalytic activity of an enzyme encoded by SEQ ID NO.:5.

11. The transgenic plant material according to claim 10 wherein the isolated DNA encodes the enzyme from positions 141 to 1688 in SEQ ID. NO.:5.

12. The transgenic plant material according to claim 9 wherein the isolated DNA encodes a marigold enzyme having the amino acid sequence of SEQ ID NO.:5.

13. A transgenic plant material comprising one or more isolated DNAs encoding marigold enzymes selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-hydroxylase and epsilon-cyclase.

14. The transgenic plant material according to claim 13 wherein the beta-cyclase is encoded by SEQ ID NO.:1, the beta-hydroxylase is encoded by SEQ ID NO.:3 and the epsilon-cyclase is encoded by SEQ ID NO.: 5.

15. The transgenic plant material according to claim 14 wherein the beta-cyclase is encoded by positions 304 to 1836 of SEQ ID NO.:1, the beta-hydroxylase is encoded by positions 51 to 923 of SEQ ID NO.:3 and the epsilon-cyclase is encoded by positions 141 to 1688 of SEQ ID NO.: 5.

16. The transgenic plant material according to claim 15 wherein the beta-hydroxylase has the amino acid sequence of SEQ ID NO.:2, the beta-cyclase has the amino acid sequence of SEQ ID NO.:4 and the epsilon-cyclase has the amino acid sequence of SEQ ID NO.: 6.

17. An isolated DNA comprising a DNA sequence encoding marigold beta-cyclase.

18. The isolated DNA according to claim 17 having the DNA sequence of SEQ ID NO.:1.

19. The isolated DNA according to claim 18 wherein the DNA sequence encodes beta-cyclase having the amino acid sequence of SEQ ID NO.:2.

20. An isolated DNA comprising a DNA sequence encoding marigold beta-hydroxylase.

21. The isolated DNA according to claim 20 having the DNA sequence of SEQ ID NO.:3.

22. The isolated DNA according to claim 21 wherein the DNA sequence encodes beta-hydroxylase having the amino acid sequence of SEQ ID NO.:4.

23. An isolated DNA comprising a DNA sequence encoding marigold epsilon-cyclase.

24. The isolated DNA according to claim 23 having the DNA sequence of SEQ ID NO.:5.

25. The isolated DNA according to claim 24 wherein the DNA sequence encodes epsilon-cyclase having the amino acid sequence of SEQ ID NO.:6.

26. A transgenic plant material containing at least one isolated DNA which produces an RNA that is antisense to a marigold enzyme selected from the group consisting of beta-cyclase, beta-hydroxylase, epsilon-hydroxylase and epsilon-cyclase.

27. The transgenic plant material according to claim 26 wherein the isolated DNA is selected from the group consisting of beta-cyclase encoded by SEQ ID NO.:1, beta-hydroxylase encoded by SEQ ID NO.:3 and epsilon-cyclase encoded by SEQ ID NO.:5.

28. The transgenic plant material according to claim 27 wherein the isolated DNA is selected from the group consisting of beta-cyclase encoded by positions 304 to 1836 of SEQ ID NO.:1, beta-hydroxylase encoded by positions 51 to 923 of SEQ ID NO.:3 and epsilon-cyclase encoded by positions 141 to 1688 of SEQ ID NO.:5.

29. A transgenic plant material containing at least one isolated marigold DNA sequence selected from the group consisting of a DNA sequence encoding an enzyme having catalytic activity of beta-hydroxylase, a DNA sequence encoding an enzyme having catalytic activity of beta-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-hydroxylase and a DNA sequence encoding an enzyme having catalytic activity of IPP isomerase wherein a first end of the DNA sequence is operably linked to a RNA promoter and a second end of the DNA sequence is operably linked to a regulatory sequence containing a polyadenylation signal, such that upon transformation, the plant material produces the enzyme encoded by the isolated DNA sequence.

30. A transgenic plant material containing at least one isolated marigold DNA sequence selected from the group consisting of a DNA sequence encoding an enzyme having catalytic activity of beta-hydroxylase, a DNA sequence encoding an enzyme having catalytic activity of beta-cyclase, a DNA sequence encoding an enzyme having catalytic

activity of epsilon-cyclase, and a DNA sequence encoding an enzyme having catalytic activity of epsilon-hydroxylase wherein a first end of the DNA sequence is operably linked to a RNA promoter and a second end of the DNA sequence is operably linked to a regulatory sequence containing a polyadenylation signal, such that upon transformation, the plant material produces an RNA antisense to a mRNA produced by the plant.

31. A transgenic plant material containing at least two marigold DNA sequences selected from the group consisting of a DNA sequence encoding an enzyme having catalytic activity of beta-hydroxylase, a DNA sequence encoding an enzyme having catalytic activity of beta-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-hydroxylase and a DNA sequence encoding an enzyme having catalytic activity of IPP isomerase wherein a first end of the DNA sequence is operably linked to a RNA promoter, and wherein at least one DNA sequence produces an RNA in an orientation antisense to a mRNA and remaining DNA sequence produces an RNA in a sense orientation such that upon transformation, the plant material produces an RNA molecule from the first recombinant DNA construct antisense to the mRNA produced by the plant and produces the enzyme encoded by the second recombinant DNA construct.

32. The transgenic plant according to any one of claims 29, 30 or 31, wherein the DNA sequence encoding the beta-cyclase is encoded by positions 304 to 1836 of SEQ ID NO.1, the DNA sequence encoding beta-hydroxylase is encoded by positions 51 to 923 of SEQ ID NO.:3, and the DNA sequence encoding the epsilon-cyclase is encoded by positions 141 to 1688 of SEQ ID NO.:5.

33. The transgenic plant according to any one of claims 29, 30 or 31 wherein the RNA promoter is a petal specific promoter.

34. The transgenic plant according to claim 33 wherein the RNA promoter is a promoter for a ketolase gene from *Arabidopsis thaliana.*

35. The transgenic plant according to claim 34 wherein the RNA promoter is a promoter for a ketolase gene from *Adonis vernalis.*

36. A method for manipulating carotenoid synthesis in a plant material, the steps comprising:

(a) providing at least one isolated marigold DNA sequence selected from the group consisting of a DNA sequence encoding an enzyme having catalytic activity of beta-hydroxylase, a DNA sequence encoding an enzyme having catalytic activity of beta-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-hydroxylase, a DNA sequence encoding an enzyme having catalytic activity of IPP isomerase wherein a first end of the DNA sequence is operably linked to a RNA promoter and a second end of the DNA sequence is operably linked to a regulatory sequence containing a polyadenylation signal;

(b) transforming plant material with the isolated DNA; and

(c) isolating the plant.

37. The method according to claim 36 wherein at least one isolated DNA is operably linked to the RNA promoter to produce an RNA that is antisense to an mRNA.

38. The method according to claim 36 wherein at least one isolated DNA is operably linked to the RNA promoter to produce the enzyme encoded by the isolated DNA sequence.

39. The transgenic plant of any one according to claims 36, 37 or 38 wherein the RNA promoter is a petal specific promoter.

40. The transgenic plant according to claim 39 wherein the RNA promoter is a promoter for a ketolase gene from *Adonis vernalis*.

41. A transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of IPP isomerase.

36. A method for manipulating carotenoid synthesis in a plant material, the steps comprising:

(a) providing at least one isolated marigold DNA sequence selected from the group consisting of a DNA sequence encoding an enzyme having catalytic activity of beta-hydroxylase, a DNA sequence encoding an enzyme having catalytic activity of beta-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-cyclase, a DNA sequence encoding an enzyme having catalytic activity of epsilon-hydroxylase, a DNA sequence encoding an enzyme having catalytic activity of IPP isomerase wherein a first end of the DNA sequence is operably linked to a RNA promoter and a second end of the DNA sequence is operably linked to a regulatory sequence containing a polyadenylation signal;

(b) transforming plant material with the isolated DNA; and

(c) isolating the plant.

37. The method according to claim 36 wherein at least one isolated DNA is operably linked to the RNA promoter to produce an RNA that is antisense to an mRNA.

38. The method according to claim 36 wherein at least one isolated DNA is operably linked to the RNA promoter to produce the enzyme encoded by the isolated DNA sequence.

39. The transgenic plant of any one according to claims 36, 37 or 38 wherein the RNA promoter is a petal specific promoter.

40. The transgenic plant according to claim 39 wherein the RNA promoter is a promoter for a ketolase gene from *Adonis vernalis*.

41. A transgenic plant material containing an isolated DNA encoding a marigold enzyme having catalytic activity of IPP isomerase.

42. The transgenic plant material according to claim 41 wherein the isolated DNA encodes a marigold enzyme having catalytic activity of an enzyme encoded by SEQ ID NO.:7.

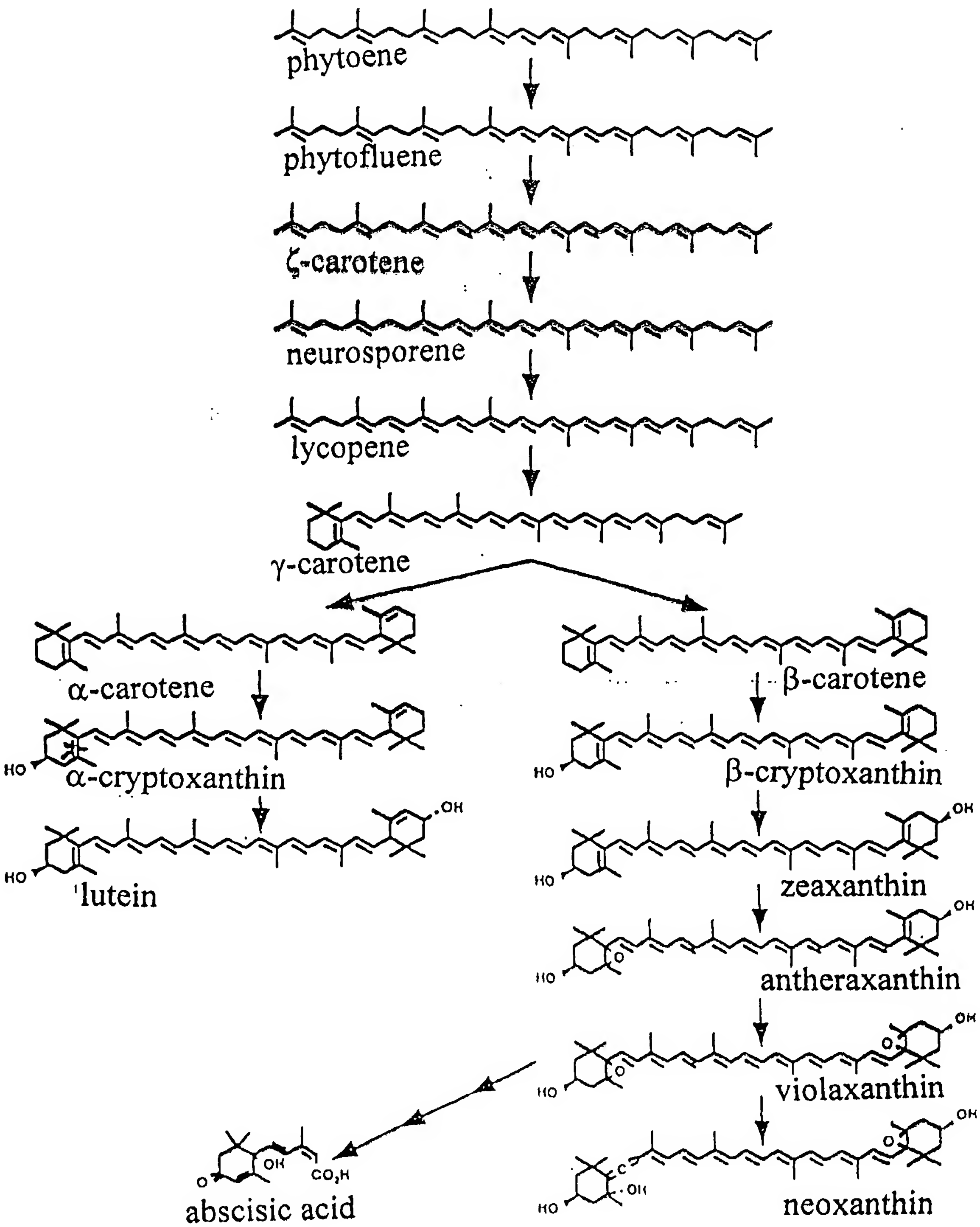
43. The transgenic plant material according to claim 42 wherein the isolated DNA encodes the enzyme from positions 101 to 796 in SEQ ID. NO.:7.

44. The transgenic plant material according to claim 43 wherein the isolated DNA encodes a marigold enzyme having the amino acid sequence of SEQ ID NO.:8.

45. An isolated DNA comprising a DNA sequence encoding marigold IPP isomerase.

46. The isolated DNA according to claim 45 having the DNA sequence of SEQ ID NO.:7.

47. The isolated DNA according to claim 46 wherein the DNA sequence encodes beta-cyclase having the amino acid sequence of SEQ ID NO.:8.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phytofluene
ζ-carotene
neurosporene
lycopene
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β-carotene
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β-cryptoxanthin
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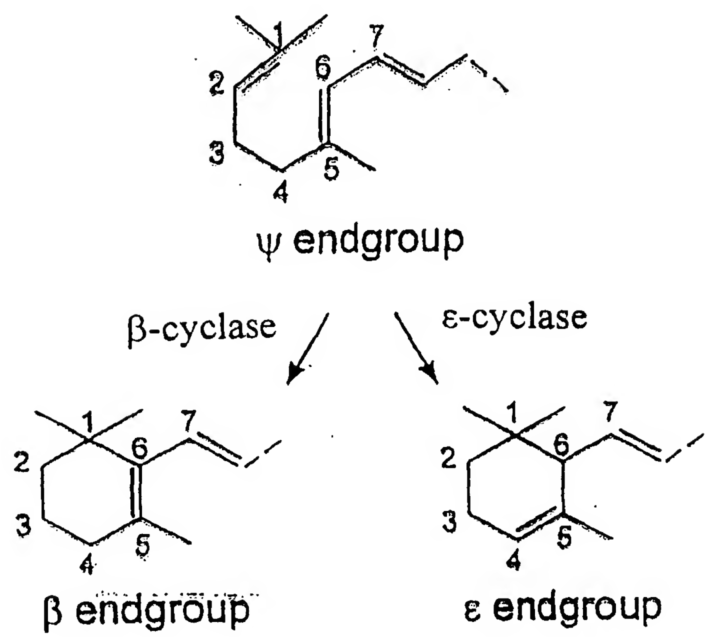
neoxanthin

Fig. 1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2
6
7
3
5
4
ψ endgroup
β-cyclase
ε-cyclase
1
7
2
6
3
5
4
β endgroup
1
7
2
6
3
5
4
ε endgroup

Fig. 2

TCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGACTTCCCATTATCC
AATCTCTCAAAACCATCAACAATTTCACCACATCATTTACCGGTAAGTCTTCATATCTTT
CAATTCTTCACAAACCCACTTCAATTCTCATCATTAATCTCATAAAGTTCATACCTTTGTT
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TATGCTTTCAGTTTTTGTTAATTGGATGTTATGGTAATTGTATGTTTTAAGTTGATTAAA
AAAAAAAAAAAAAAA

Fig. 3

MDTFLRTYNSFEFVHPSNKFAGNLNNLNQLNQSKSQFQDFRFGPKKSQFKLGQKYCVK
ASSSALLELVPEIKKENLDFDLPMYDPSRNVVVDLVVVGGGPSGLAVAQQVSEAGLTVCSI
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Fig. 4

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Fig. 5

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Fig. 6

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Fig. 7

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PYVEMFGTFALSVGAAVGMEYWARWAHEALWHASLWHMHESHHKPREGPFELNDVFA
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Fig. 8

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Fig. 9

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Fig. 10

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agaatcatta ctaacaatca atg agt atg aga gct gga cac atg acg gca aca 173
                      Met Ser Met Arg Ala Gly His Met Thr Ala Thr
                      1               5                   10

atg gcg gct ttt aca tgc cct agg ttt atg act agc atc aga tac acg   221
Met Ala Ala Phe Thr Cys Pro Arg Phe Met Thr Ser Ile Arg Tyr Thr
            15                  20                  25

aag caa att aag tgc aac gct gct aaa agc cag cta gtc gtt aaa caa   269
Lys Gln Ile Lys Cys Asn Ala Ala Lys Ser Gln Leu Val Val Lys Gln
        30                  35                  40

gag att gag gag gaa gaa gat tat gtg aaa gcc ggt gga tcg gag ctg   317
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Glu Ile Glu Glu Glu Glu Asp Tyr Val Lys Ala Gly Gly Ser Glu Leu
45 50 55

ctt ttt gtt caa atg caa cag aat aag tcc atg gat gca cag tct agc 365
Leu Phe Val Gln Met Gln Gln Asn Lys Ser Met Asp Ala Gln Ser Ser
60 65 70 75

cta tcc caa aag ctc cca agg gta cca ata gga gga gga gga gac agt 413
Leu Ser Gln Lys Leu Pro Arg Val Pro Ile Gly Gly Gly Gly Asp Ser
80 85 90

aac tgt ata ctg gat ttg gtt gta att ggt tgt ggt cct gct ggc ctt 461
Asn Cys Ile Leu Asp Leu Val Val Ile Gly Cys Gly Pro Ala Gly Leu
95 100 105

gct ctt gct gga gaa tca gcc aag cta ggc ttg aat gtc gca ctt atc 509
Ala Leu Ala Gly Glu Ser Ala Lys Leu Gly Leu Asn Val Ala Leu Ile
110 115 120

ggc cct gat ctt cct ttt aca aat aac tat ggt gtt tgg gag gat gaa 557
Gly Pro Asp Leu Pro Phe Thr Asn Asn Tyr Gly Val Trp Glu Asp Glu
125 130 135

ttt ata ggt ctt gga ctt gag ggc tgt att gaa cat gtt tgg cga gat 605
Phe Ile Gly Leu Gly Leu Glu Gly Cys Ile Glu His Val Trp Arg Asp
140 145 150 155

act gta gta tat ctt gat gac aac gat ccc att ctc ata ggt cgt gcc 653
Thr Val Val Tyr Leu Asp Asp Asn Asp Pro Ile Leu Ile Gly Arg Ala
160 165 170

tat gga cga gtt agt cgt gat tta ctt cac gag gag ttg ttg act agg 701
Tyr Gly Arg Val Ser Arg Asp Leu Leu His Glu Glu Leu Leu Thr Arg
175 180 185

tgc atg gag tca ggc gtt tca tat ctg agc tcc aaa gtg gaa cgg att 749
Cys Met Glu Ser Gly Val Ser Tyr Leu Ser Ser Lys Val Glu Arg Ile
190 195 200

act gaa gct cca aat ggc cta agt ctc ata gag tgt gaa ggc aat atc 797
Thr Glu Ala Pro Asn Gly Leu Ser Leu Ile Glu Cys Glu Gly Asn Ile
205 210 215

aca att cca tgc agg ctt gct act gtc gct tct gga gca gct tct gga 845
Thr Ile Pro Cys Arg Leu Ala Thr Val Ala Ser Gly Ala Ala Ser Gly
220 225 230 235

aaa ctt ttg cag tat gaa ctt ggc ggt ccc cgt gtt tgc gtt caa aca 893
Lys Leu Leu Gln Tyr Glu Leu Gly Gly Pro Arg Val Cys Val Gln Thr
240 245 250

gct tat ggt ata gag gtt gag gtt gaa agc ata ccc tat gat cca agc 941
Ala Tyr Gly Ile Glu Val Glu Val Glu Ser Ile Pro Tyr Asp Pro Ser
255 260 265

cta atg gtt ttc atg gat tat aga gac tac acc aaa cat aaa tct caa 989
Leu Met Val Phe Met Asp Tyr Arg Asp Tyr Thr Lys His Lys Ser Gln
270 275 280

tca cta gaa gca caa tat cca aca ttt ttg tat gtc atg cca atg tct 1037
Ser Leu Glu Ala Gln Tyr Pro Thr Phe Leu Tyr Val Met Pro Met Ser
285 290 295

cca act aaa gta ttc ttt gag gaa act tgt ttg gct tca aaa gag gcc 1085
Pro Thr Lys Val Phe Phe Glu Glu Thr Cys Leu Ala Ser Lys Glu Ala
300 305 310 315

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atg cct ttt gag tta ttg aag aca aaa ctc atg tca aga tta aag act     1133
Met Pro Phe Glu Leu Leu Lys Thr Lys Leu Met Ser Arg Leu Lys Thr
                320                 325                 330

atg ggg atc cga ata acc aaa act tat gaa gag gaa tgg tca tat att     1181
Met Gly Ile Arg Ile Thr Lys Thr Tyr Glu Glu Glu Trp Ser Tyr Ile
            335                 340                 345

cca gta ggt gga tcc tta cca aat acc gag caa aag aac ctt gca ttt     1229
Pro Val Gly Gly Ser Leu Pro Asn Thr Glu Gln Lys Asn Leu Ala Phe
        350                 355                 360

ggt gct gct gct agc atg gtg cat cca gcc aca gga tat tcg gtt gta     1277
Gly Ala Ala Ala Ser Met Val His Pro Ala Thr Gly Tyr Ser Val Val
    365                 370                 375

aga tca ctg tca gaa gct cct aat tat gca gca gta att gca aag att     1325
Arg Ser Leu Ser Glu Ala Pro Asn Tyr Ala Ala Val Ile Ala Lys Ile
380                 385                 390                 395

tta ggg aaa gga aat tca aaa cag atg ctt gat cat gga aga tac aca     1373
Leu Gly Lys Gly Asn Ser Lys Gln Met Leu Asp His Gly Arg Tyr Thr
                400                 405                 410

acc aac atc tca aag caa gct tgg gaa aca ctt tgg ccc ctt gaa agg     1421
Thr Asn Ile Ser Lys Gln Ala Trp Glu Thr Leu Trp Pro Leu Glu Arg
            415                 420                 425

aaa aga cag aga gca ttc ttt ctc ttt gga tta gca ctg att gtc cag     1469
Lys Arg Gln Arg Ala Phe Phe Leu Phe Gly Leu Ala Leu Ile Val Gln
        430                 435                 440

atg gat att gag ggg acc cgc aca ttc ttc cgg act ttc ttc cgc ttg     1517
Met Asp Ile Glu Gly Thr Arg Thr Phe Phe Arg Thr Phe Phe Arg Leu
    445                 450                 455

ccc aca tgg atg tgg tgg ggg ttt ctt gga tct tcg tta tca tca act     1565
Pro Thr Trp Met Trp Trp Gly Phe Leu Gly Ser Ser Leu Ser Ser Thr
460                 465                 470                 475

gac ttg ata ata ttt gcg ttt tac atg ttt atc ata gca ccg cat agc     1613
Asp Leu Ile Ile Phe Ala Phe Tyr Met Phe Ile Ile Ala Pro His Ser
                480                 485                 490

ctg aga atg ggt ctg gtt aga cat ttg ctt tct gac ccg aca gga gga     1661
Leu Arg Met Gly Leu Val Arg His Leu Leu Ser Asp Pro Thr Gly Gly
            495                 500                 505

aca atg tta aaa gcg tat ctc acg ata taaataactc tagtcgcgat           1708
Thr Met Leu Lys Ala Tyr Leu Thr Ile
        510                 515

cagtttagat tataggcaca tcttgcatat atatatgtat aaaccttatg tgtgctgtat 1768

ccttacatca acacagtcat taattgtatt tcttggggta atgctgatga agtattttca 1828

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Cys Pro Arg Phe Met Thr Ser Ile Arg Tyr Thr Lys Gln Ile Lys Cys
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Asn Ala Ala Lys Ser Gln Leu Val Val Lys Gln Glu Ile Glu Glu Glu
        35                  40                  45

Glu Asp Tyr Val Lys Ala Gly Gly Ser Glu Leu Leu Phe Val Gln Met
    50                  55                  60

Gln Gln Asn Lys Ser Met Asp Ala Gln Ser Ser Leu Ser Gln Lys Leu
65                  70                  75                  80

Pro Arg Val Pro Ile Gly Gly Gly Gly Asp Ser Asn Cys Ile Leu Asp
                85                  90                  95

Leu Val Val Ile Gly Cys Gly Pro Ala Gly Leu Ala Leu Ala Gly Glu
            100                 105                 110

Ser Ala Lys Leu Gly Leu Asn Val Ala Leu Ile Gly Pro Asp Leu Pro
        115                 120                 125

Phe Thr Asn Asn Tyr Gly Val Trp Glu Asp Glu Phe Ile Gly Leu Gly
    130                 135                 140

Leu Glu Gly Cys Ile Glu His Val Trp Arg Asp Thr Val Val Tyr Leu
145                 150                 155                 160

Asp Asp Asn Asp Pro Ile Leu Ile Gly Arg Ala Tyr Gly Arg Val Ser
                165                 170                 175

Arg Asp Leu Leu His Glu Glu Leu Leu Thr Arg Cys Met Glu Ser Gly
            180                 185                 190

Val Ser Tyr Leu Ser Ser Lys Val Glu Arg Ile Thr Glu Ala Pro Asn
        195                 200                 205

Gly Leu Ser Leu Ile Glu Cys Glu Gly Asn Ile Thr Ile Pro Cys Arg
    210                 215                 220

Leu Ala Thr Val Ala Ser Gly Ala Ala Ser Gly Lys Leu Leu Gln Tyr
225                 230                 235                 240

Glu Leu Gly Gly Pro Arg Val Cys Val Gln Thr Ala Tyr Gly Ile Glu
                245                 250                 255

Val Glu Val Glu Ser Ile Pro Tyr Asp Pro Ser Leu Met Val Phe Met
            260                 265                 270

Asp Tyr Arg Asp Tyr Thr Lys His Lys Ser Gln Ser Leu Glu Ala Gln
        275                 280                 285

Tyr Pro Thr Phe Leu Tyr Val Met Pro Met Ser Pro Thr Lys Val Phe
    290                 295                 300

Phe Glu Glu Thr Cys Leu Ala Ser Lys Glu Ala Met Pro Phe Glu Leu
305                 310                 315                 320

Leu Lys Thr Lys Leu Met Ser Arg Leu Lys Thr Met Gly Ile Arg Ile
                325                 330                 335

Thr Lys Thr Tyr Glu Glu Glu Trp Ser Tyr Ile Pro Val Gly Gly Ser
            340                 345                 350
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Leu Pro Asn Thr Glu Gln Lys Asn Leu Ala Phe Gly Ala Ala Ala Ser
355 360 365

Met Val His Pro Ala Thr Gly Tyr Ser Val Val Arg Ser Leu Ser Glu
370 375 380

Ala Pro Asn Tyr Ala Ala Val Ile Ala Lys Ile Leu Gly Lys Gly Asn
385 390 395 400

Ser Lys Gln Met Leu Asp His Gly Arg Tyr Thr Thr Asn Ile Ser Lys
405 410 415

Gln Ala Trp Glu Thr Leu Trp Pro Leu Glu Arg Lys Arg Gln Arg Ala
420 425 430

Phe Phe Leu Phe Gly Leu Ala Leu Ile Val Gln Met Asp Ile Glu Gly
435 440 445

Thr Arg Thr Phe Phe Arg Thr Phe Phe Arg Leu Pro Thr Trp Met Trp
450 455 460

Trp Gly Phe Leu Gly Ser Ser Leu Ser Ser Thr Asp Leu Ile Ile Phe
465 470 475 480

Ala Phe Tyr Met Phe Ile Ile Ala Pro His Ser Leu Arg Met Gly Leu
485 490 495

Val Arg His Leu Leu Ser Asp Pro Thr Gly Gly Thr Met Leu Lys Ala
500 505 510

Tyr Leu Thr Ile
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caaaaacaac tcaaatctcc tccgtcgctc ttactccgcc atg ggt gac gac tcc 115
Met Gly Asp Asp Ser
1 5

ggc atg gat gct gtt cag cga cgt ctc atg ttt aac gat gaa tgc att 163
Gly Met Asp Ala Val Gln Arg Arg Leu Met Phe Asn Asp Glu Cys Ile
10 15 20

ttg gtg gat gag tgt gac aat gtg gtg gga cat gat acc aaa tac aat 211
Leu Val Asp Glu Cys Asp Asn Val Val Gly His Asp Thr Lys Tyr Asn
25 30 35

tgt cac ttg atg gag aag att gaa aca ggt aaa atg ctg cac aga gca 259
Cys His Leu Met Glu Lys Ile Glu Thr Gly Lys Met Leu His Arg Ala
40 45 50

ttc agc gtt ttt cta ttc aat tca aaa tac gag tta ctt ctt cag caa 307
Phe Ser Val Phe Leu Phe Asn Ser Lys Tyr Glu Leu Leu Leu Gln Gln
55 60 65

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cgg tct gca acc aag gtg aca ttt cct tta gta tgg acc aac acc tgt    355
Arg Ser Ala Thr Lys Val Thr Phe Pro Leu Val Trp Thr Asn Thr Cys
 70                 75                  80                  85

tgc agc cat cca ctc tac aga gaa tcc gag ctt gtt ccc gaa aac gcc    403
Cys Ser His Pro Leu Tyr Arg Glu Ser Glu Leu Val Pro Glu Asn Ala
                90                  95                 100

ctt gga gta aga aat gct gca cag agg aag ctg ttg gat gaa ctc ggt    451
Leu Gly Val Arg Asn Ala Ala Gln Arg Lys Leu Leu Asp Glu Leu Gly
            105                 110                 115

atc cct gct gaa gat gtt ccc gtt gat cag ttt act cct tta ggt cgc    499
Ile Pro Ala Glu Asp Val Pro Val Asp Gln Phe Thr Pro Leu Gly Arg
        120                 125                 130

atg ctc tac aag gct cca tct gat gga aag tgg gga gaa cat gaa ctt    547
Met Leu Tyr Lys Ala Pro Ser Asp Gly Lys Trp Gly Glu His Glu Leu
    135                 140                 145

gac tac cta ctt ttc ata gtg aga gac gtt gct gta aac ccg aac cca    595
Asp Tyr Leu Leu Phe Ile Val Arg Asp Val Ala Val Asn Pro Asn Pro
150                 155                 160                 165

gat gaa gtg gcg gat atc aaa tat gtg aac caa gaa gag tta aag gag    643
Asp Glu Val Ala Asp Ile Lys Tyr Val Asn Gln Glu Glu Leu Lys Glu
                170                 175                 180

ctg cta agg aaa gca gat gcg ggg gag gag ggt ttg aag ctg tct cca    691
Leu Leu Arg Lys Ala Asp Ala Gly Glu Glu Gly Leu Lys Leu Ser Pro
            185                 190                 195

tgg ttc agg tta gtg gtt gat aac ttc ttg ttc aag tgg tgg gat cat    739
Trp Phe Arg Leu Val Val Asp Asn Phe Leu Phe Lys Trp Trp Asp His
        200                 205                 210

gtg caa aag gtt aca ctc act gaa gca att gat atg aaa acc ata cac    787
Val Gln Lys Val Thr Leu Thr Glu Ala Ile Asp Met Lys Thr Ile His
    215                 220                 225

aag ctg ata tagaaacaca ccctcaacgg aaaagttcaa gcctaataat            836
Lys Leu Ile
230

tcgggttggg tcgggtctac catcaattgt ttttttcttt taagaagttt taatctctat 896

ttgagcatgt tgattcttgt cttttgtgtg taagattttg ggtttcgttt cagttgtaat 956

aatgaaccat tgatggtttg caatttcaag ttcctatcga caaaaaaaaa aaaaaaaaaa 1016

actc                                                              1020


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 1               5                  10                  15

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            20                  25                  30
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Asp Thr Lys Tyr Asn Cys His Leu Met Glu Lys Ile Glu Thr Gly Lys
        35                  40                  45

Met Leu His Arg Ala Phe Ser Val Phe Leu Phe Asn Ser Lys Tyr Glu
    50                  55                  60

Leu Leu Leu Gln Gln Arg Ser Ala Thr Lys Val Thr Phe Pro Leu Val
65                  70                  75                  80

Trp Thr Asn Thr Cys Cys Ser His Pro Leu Tyr Arg Glu Ser Glu Leu
                85                  90                  95

Val Pro Glu Asn Ala Leu Gly Val Arg Asn Ala Ala Gln Arg Lys Leu
            100                 105                 110

Leu Asp Glu Leu Gly Ile Pro Ala Glu Asp Val Pro Val Asp Gln Phe
        115                 120                 125

Thr Pro Leu Gly Arg Met Leu Tyr Lys Ala Pro Ser Asp Gly Lys Trp
    130                 135                 140

Gly Glu His Glu Leu Asp Tyr Leu Leu Phe Ile Val Arg Asp Val Ala
145                 150                 155                 160

Val Asn Pro Asn Pro Asp Glu Val Ala Asp Ile Lys Tyr Val Asn Gln
                165                 170                 175

Glu Glu Leu Lys Glu Leu Leu Arg Lys Ala Asp Ala Gly Glu Glu Gly
            180                 185                 190

Leu Lys Leu Ser Pro Trp Phe Arg Leu Val Val Asp Asn Phe Leu Phe
        195                 200                 205

Lys Trp Trp Asp His Val Gln Lys Val Thr Leu Thr Glu Ala Ile Asp
    210                 215                 220

Met Lys Thr Ile His Lys Leu Ile
225                 230
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